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We have focussed our efforts on investigating the mechanisms by which antisense to type I insulin-like growth factor receptor (IGF-IR) or antisense to transforming growth factor β (TGFβ) reduces tumorigenicity in human and murine breast cancer cells. MDA-MB-435S human breast cancer cells expressing antisense IGF-IR enhanced apoptosis *in vitro*, which could contribute to the reduced tumorigenicity observed *in vivo*. A dramatic reduction in tumorigenesis and an absence of lung metastasis was seen in 3 models of immune compromised mice (nude, scid, scid-beige) injected with antisense IGF-IR transfected MDA-MB-435S cells. All mice injected with parental or control transfected cells developed large tumors. However, only the scid beige animals consistently exhibited pulmonary metastases indicating that this animal is the most suitable for studying the metastatic behavior of these human breast cancer cells. We have established baseline parameters for IL-2, IL-4, IL-5 and interferon γ in EMT6 murine breast cancer cells and in antisense IGF-IR and antisense TGFβ transfectants. Spleen cells from syngeneic mice injected with EMT6 cells expressing antisense TGF-β show increased cytotoxicity. The percentage of apoptotic cells from tumors excised from these animals is significantly reduced.

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Judith Han
Principal Investigator's Signature

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Introduction:

In the United States approximately 183,000 new cases of breast cancer were diagnosed in 1995, with the number of deaths estimated at around 46,000 (Wingo et al., 1995). Ninety percent of patients dying with breast cancer have osteolytic bone metastases (Yoneda et al., 1994). Therefore, it is urgent to find treatments that will result in a cure. Current standard treatments include chemotherapy, irradiation and bone marrow transplantation. However, immunological approaches such as adoptive immunotherapy and rationally designed biological agents that attack specific proteins unique to the malignant cancer cells remain to be developed (Lippman, 1993). A number of proteins such as the insulin-like growth factors (IGFs), insulin-like growth factor receptors (IGF-IRs), and transforming growth factors (TGFs) have been shown to participate in the growth of breast cancer cells (Cullen et al., 1990; Dickson & Lippman, 1995). We are applying our experience in treating and preventing rat glioblastoma (Trojan et al., 1992; Trojan et al., 1993) and murine teratocarcinoma (Trojan et al., 1994) to develop a model for breast cancer.

To assign a role of a gene product, one relies on naturally occurring mutants that fail to express the gene in normal fashion. Generation of cellular mutants has limited applicability due to the diploid nature of most genes and the lack of adequate mutant selection. Our experimental approach for breast cancer centers around the use of antisense RNA expression to produce phenocopies of a null mutation of the type I insulin-like growth factor receptor (IGF-IR) and transforming growth factors (TGFs). The antisense strategy bypasses inherent limitations of functional studies dependent upon natural mutant cells or artificially mutagenized cells. Inhibition of IGF receptors or TGFs by antisense provides a direct approach for assessing their contribution to the tumorigenic phenotype of cancer cells both in culture and *in vivo*. The antisense RNA will either hybridize to the endogenous mRNA or disrupt its transcription or processing, thereby preventing the synthesis of protein product (Izant and Weintraub, 1985). We (Johnson et al., 1991) and others (Keiss et al., 1989) have reported that rat glioma C6 cells express high levels of IGF-I which is enhanced when the cells are grown in serum-free medium. We have observed that rats injected with transfected glioma cells that express antisense IGF-I RNA and which lack IGF-I protein continue to remain tumor-free for more than two years. In contrast, rats injected with parental (non-transfected) glioma cells consistently develop large tumors within a few weeks post-injections. These data demonstrate a transformation role for IGF-I, highlighting the fact that transformed phenotypes may arise through unexpected molecular mechanisms. It represents a clear example of a dominant and essential role for a growth factor in malignant transformation.

Antisense strategy has been applied successfully to a growing set of genes in both cultured cells and transgenic animals (Katsuri et al., 1988; Munir et al., 1990). However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression (Munir et al., 1990; Khokha et al., 1989). We (Johnson et al., 1991; Trojan et al., 1992) have demonstrated effective antisense inhibition of endogenous IGF-I transcripts in C6 glioblastoma cells by Northern analysis and inhibition of protein by immunocytochemistry.

Antisense IGF-I transcripts were prominently localized to the nuclei by *in situ* hybridization supporting an intranuclear mechanism for antisense RNA-mediated inhibition, in agreement with observations made by others (Kim and Wold, 1985: Stout and Caskey, 1990; Corenlissen, 1989; Munroe, 1988). A finding in our study was accumulation of mononuclear infiltrates, with a predominance of lymphoid cells, at sites of injection of antisense IGF-I transfected glioma cells before lesions disappeared (Trojan et al., 1992). The majority of cells were CD8 positive, suggesting antisense IGF-I inhibition rendered glioma cells highly immunogenic, and hence, loss of tumorigenicity had an immune basis. This is further supported by a recent study which shows that glioblastoma cells transfected with antisense to IGF-I demonstrate a significant increase in major histocompatibility complex-I (MHC-I) when compared to untransfected cells by FACS techniques (Trojan et al., 1996; Shevelev et al., 1997). We have shown that prior injection of the antisense IGF-I transfected glioma cells can prevent development of glioblastoma upon subsequent challenge with parental glioma cells. Injection of the genetically engineered glioma cells into rats with established glioblastomas cures the rats. The findings are consistent with a role for the host immune response in the anti-tumor therapeutic effects (Trojan et al., 1993; Johnson et al., 1993). We have also utilized the antisense gene therapy approach to successfully treat mice with teratocarcinoma (Trojan et al., 1994).

Prior research has focused on the potential role of the IGFs as growth factors driving the proliferation of tumor cells that produce them. This has led to attempts to inhibit growth of such tumor cells with anti-IGF-I or anti-IGF-II antibodies. While some growth inhibition has been claimed using these antibodies *in vitro* (Minuto et al., 1987; Huff et al., 1986; Blatt et al., 1984) and *in vivo* (Gansler et al., 1989), tumor development could not be completely blocked using the anti-IGF antibodies. Moreover, since intracrine mechanisms (Heldin and Westermark, 1989) may well be involved here, there is no certainty that the effects of anti-IGF-I antibodies will parallel those of antisense IGF-I RNA in altering tumor immunogenicity in an immunologically intact animal.

Tumors that arise *de novo* are poorly immunogenic, thereby escaping host anti-tumor responses (Hewitt et al., 1979). Our studies provide a potential therapeutic approach toward enchancing tumor immunogenicity based upon antisense gene transfer. Alternative approaches based upon sense gene transfer have been reported for enhancing tumor cell immunogenicity (Fearon et al., 1988). However, loss of tumorigenicity of transfected tumor cells and tumor prevention using these cells were incomplete. The second approach involves enhancement of tumor immunogenicity by transferring into cells, genes expressing soluble cytokines, such as interleukin-2 (Fearon and Vogelstein, 1990) and interleukin-4 (Tepper et al., 1989; Golumbek et al., 1991). Results with this approach have been more promising than those with foreign antigen transfer. Indeed, it has been demonstrated that production of IL-2 by the mouse mammary sarcoma EMT6 transfected with a murine IL-2 cDNA is able to elicit rejection of the tumor, and that the rejection is associated with development of cytotoxic T-lymphocytes that can lyse the parental tumor (McAdam et al., 1994). In addition, human breast cancer cells MDA-MB-435 transduced with human IL-2 did not form tumors when injected into the mammary fat pad of nude mice (Su et al., 1994). Furthermore, it was shown that transfecting cultured murine melanoma cells with the co-stimulator B7 evoked an

effective immune response which results in regression of the existing tumor in syngeneic animals (Townsend and Allison, 1993; Chen et al., 1992).

Many primary tumors and cell lines from tumors produce large amounts of IGFs and IGF receptors (Antoinades et al., 1992; Roholl et al., 1990; Williams et al., 1989; Gansler et al., 1988; Culouscou et al., 1987; Macaulay et al., 1990; Jing et al., 1991). Most important for this proposal are breast carcinomas that have also been shown to express IGFs and IGF receptors (Huff et al., 1986; Yee et al., 1988; Foekins et al., 1989; Brunner et al., 1990). In order to postulate an autocrine or paracrine role for IGFs in breast cancer, breast cancer cells must have appropriate receptors for these ligands. Cullen et al. (1991) examined breast cancer cell lines and tumor samples for mRNA expression of the insulin receptor as well as the type I and type II IGF receptors. All cell lines examined by this group expressed mRNA for these receptors. In addition, 6 of 7 breast tumor biopsy specimens expressed type I IGF receptor mRNA. The monoclonal antibody (αIR3) which blocks binding to the type I IGF receptor also blocked the mitogenic effects of both IGF-I and IGF-II, but not insulin. The αIR3 antibody was also able to block greater than 80% of radiolabeled IGF-II binding. Furthermore, αIR3 administered at the time of tumor cell inoculation could inhibit MDA-231 tumor formation in athymic mice which suggests that blockade of the IGF-IR can inhibit the growth of some breast cancer cells *in vivo* (Arteaga et al., 1989; Arteaga, 1992). This indicates that although both type I and type II IGF receptors are expressed in breast cancer cells, the mitogenic response to both IGF-I and IGF-II is mediated by the type I receptor (Cullen et al., 1990). It is likely that IGFs and the type-I IGF receptor play a pivotal role in the tumorigenicity of breast carcinoma. Most breast tumors express IGF receptors, therefore it is important to ascertain whether the inhibition of IGF-IR gene expression by an antisense approach can alter the tumorigenic potential of breast cancer cells. For this reason, we have carried out studies to determine the effect of blocking the expression of IGF-IR using antisense strategy in the human breast cancer cell line, MDA-MB-435S which was isolated from a patient with metastatic disease. We focused our efforts on blocking the expression of the IGF-IR based on a recent report from our laboratory (Rininsland et al., 1997, Appendix III). This study showed that IGF-I is suppressed in C6 rat glioblastoma cells transfected with either antisense to IGF-IR or by an IGF-IR purine triplex expression vector. These data suggested that one common mechanism could account for decreased tumorigenicity of the C6 cells regardless of whether IGF-I or IGF-IR expression is inhibited. The inhibition of IGF-I by suppression of IGF-IR by two independent approaches indicates that by targeting IGF-IR it is possible to inhibit the expression of the ligand as well as the receptor. Therefore, it is possible that targeting the IGF-IR in breast cancer cells with antisense to the receptor could also inhibit the expression of endogenous ligands such as IGFs and IGF-like species that are expressed by certain tumor cells.

Breast cancer cells appear to possess certain intrinsic properties that facilitate the development of bone metastases. Almost all patients dying of breast cancer or with advanced breast cancer have bone metastases (Yoneda et al., 1994). Breast cancer cells may prefer bone to other organs because of the growth factors in the microenvironment. Bone has been shown to be a major source of IGFs, with humans having the highest concentration of total skeletal somatomedins of all species studied (Bautista et al., 1990). Indeed, IGF-II is the

most abundant growth factor stored in bone matrix. Breast cancer cells have been shown to express type I and type II IGF receptors (Cullen et al., 1990). A recent study has demonstrated that cells expressing IGF-IR antisense RNA lost their ability to metastasize spontaneously to the liver or lung from primary subcutaneous tumors and could not colonize these organs, even when inoculated directly into their microvasculature (Long et al., 1995). These results implicate IGF-IR in the control of tumor growth and show that IGF-IR can play a crucial role in the regulation of tumor cell potential to disseminate and form metastases in secondary organs. Therefore, targeting breast tumor cells with an antisense IGF-IR strategy could provide an effective antimetastatic therapy for this disease.

Transforming growth factors (TGFs) are polypeptides that have important regulatory roles in angiogenesis, embryogeneis, inflammation and immunosuppression within both normal and transformed cells and tissues (MacCallum et al., 1994). The presence of transcripts for TGF-βs has been reported for rodent and human breast cancer cell lines (McAdam et al., 1994; Arrick et al., 1994). Furthermore, the expression of TGF-β in fresh human tumor tissue indicated that the majority of tumors expressing all three isoforms of TGF-β were derived from patients who had lymph node metastases, thereby suggesting a role for TGF-β in mammary cancer metastasis (MacCallum et al., 1994). Studies with an inbred rat model of mammary adenocarcinoma show that brief exposure of the tumor cells to TGF-β results in enhanced ability of these cells to form lung colonies when injected *in vivo* (Welch et al., 1990).

Our experimental design for breast tumors and metastases provides for an efficient coupled *in vitro/in vivo* assay system to determine the role of certain growth factors in breast cancer tumor types that may be responsible for down-modulating tumor immunogenicity or other mechanisms thereby bringing about tumor regression. Since IGF receptors or TGF-β are expressed by a number of breast cancers, it is important to ascertain whether such tumors, upon inhibition of one or more of these genes, can be correlated with an analogous induction of an immunogenic phenotype or other mechanisms that may inhibit metastasis. For this reason, we have focused our studies on blocking the expression of IGF-IR and TGF-β2 in human breast cancer cells, MDA-MB-435S and murine breast cancer cells, EMT6 using antisense expression plasmids.

Published Data:

Summary of work presented at the Department of Defense Breast Cancer Research Meeting *"Era of Hope"*, Oct 31-Nov 4, 1997, See Abstract (Appendix II).

Our proposal aimed to develop an approach that employs gene therapy for human breast tumors. We focused our efforts on targeting the type I insulin like growth factor receptor and transforming growth factor β in human and murine breast cancer cells. The ultimate aim was to develop a database supporting the hypothesis that growth factors and their receptors are involved in the tumorigenicity of certain breast cancers. We have carried out experiments on a highly metastatic human breast cancer cell line, MDA-MB-435S and a murine breast cancer cell line, EMT6.

The MDA-MB-435S human breast cancer cells express the type I insulin like growth factor receptor, therefore we transfected these cells with a plasmid carrying the IGF-IR antisense construct. Transfection of the MDA-MB-435S cells with the IGF-IR antisense construct resulted in a significant inhibition of the endogenous IGF-IR. Antisense IGF-IR transfected MDA-MB-435S cell clones showed a decrease in growth rate and colony formation, respectively. Moreover, a dramatic reduction or absence of tumor growth occurred in nude mice injected with the MDA-MB-435S cells transfected with the antisense IGF-IR construct. **A manuscript is in preparation**.

The murine breast cancer cell line, EMT6, has a syngeneic animal which provided an *in vivo* system for analyzing the effect of blocking expression of growth factors and their receptors as well as for assessing the host immune response. We found that EMT6 murine breast cancer cells express IGF-IR and TGF-β. EMT6 cell clones carrying the antisense IGF-IR construct showed a decrease in growth rate that correlated with the level of IGF-IR inhibition. A dramatic inhibition of tumor growth in both nude mice and syngeneic Balb/C mice was observed in animals injected with EMT6 cell clones that expressed the antisense for IGF-IR. The EMT6 cells also express TGF-βs which play a role in mammary tumor formation as well as metastasis. When we injected EMT6 cells transfected with antisense TGF-β2 into nude mice there was no difference in tumor size compared to control transfected cells. However, when EMT6 cells carrying antisense TGF-β were injected into the syngeneic Balb/C mice there was a dramatic reduction in tumor size when compared to control transfected EMT6 cells indicating that a functional immune system is a prerequisite for tumor inhibition to occur with antisense to TGF-β.

To date we have shown that the type I insulin-like growth factor receptor and transforming growth factor β play important roles in breast cancer tumorigenesis. Our studies suggest that the antisense gene therapy approach that targets specific growth factors and their receptors could provide a basis for the development of new biologically targeted methods for treatment of human breast cancer.

Unpublished Data

Body:

In the third year of this project, we have continued to make progress in several of the proposed goals as well as in the additional areas that we presented in our previous progress reports. In parallel with the studies we proposed in the application, we are utilizing syngeneic animal models as well as a metastatic human breast cancer model (Price and Zhang, 1989/1990, Price et al., 1990) to further elucidate the effects of our antisense strategy on tumorigenesis and metastatic processes.

A number of growth factors and their receptors are involved in tumorigenesis and influence the metastatic potential of cancer cells. Breast cancer cell lines and tumor samples have been shown to express IGF receptor (Cullen et al., 1991). Studies have shown that blockade of the type I IGF receptor with the monoclonal antibody αIR3 can reduce the rate of cell growth of MCF-7 breast cancer cells (Rohlik et al., 1987) as well as inhibit formation of MDA-MB-231 breast tumors grown in nude mice (Arteaga et al., 1989).

Transforming growth factor-β is secreted by a variety of cell types often associated with metastasizing tumor cells (Welch et al., 1990). Indeed, production of TGF-β seems to increase with breast cancer where this overproduction could contribute to aberrant tumor-host interactions (Dickson and Lippman, 1995). We have shown that EMT6 cells transfected with antisense TGF-β2 resulted in a reduction in tumor weight in syngeneic Balb/C mice but not in nude mice, underlining the important role of the immune system in the inhibition of tumors by antisense TGF-β2.

We have focused our efforts in investigating the effectiveness of antisense IGF-IR in reducing tumorigenicity and metastasis in human breast cancer cells *in vitro* and *in vivo* (using the MDA-MB-435S cells in nude mice as a model). Furthermore, we carried out experiments with EMT6 cells in nude mice and syngeneic Balb/c mice to elucidate the possible role of the immune system in reducing the tumorigencity of the antisense IGF-IR transfected cells and the antisense TGF-β transfected cells. Here, we are reporting findings over the past year that we have obtained with these two cell lines.

Construct Preparation:

We have cloned the antisense IGF-IR into a tetracycline inducible vector pBI-G in order to achieve a tight and regulatory control of antisense gene expression. Optimal *lacZ* staining and electroporation conditions were established by transient transfection of EMT6 cells with a control vector that constitutively expresses the *lacZ* gene. Staining of untransfected EMT6 cells by β-galactosidase showed less than 0.1% positive cells, whereas 50-70% of the cells transfected with the control vector displayed positive staining.

We have stably transfected the pTet-On regulator plasmid into EMT6 cells and isolated 45 clones that are resistant to 1 mg/ml neomycin. All 45 clones have been screened for low background and high doxycycline induction by transient transfections using pBI-G control plasmid vector containing the *lacZ* gene and assaying for cells that are stained blue. While all 45 clones showed low background expression of the *lacZ* gene, they have failed to demonstrate a significant increase in the *lacZ* gene expression at concentrations of doxycycline ranging from 0.1-1 μg/ml. We were advised by the technical field service personnel from Clonetech that it is necessary to screen more clones (at least 100) in order to obtain a few highly inducible clones. Therefore, we are presently isolating additional transfected clones that are neomycin resistant and testing them for inducibility with doxycycline. We have also cloned the 0.7 kb antisense IGF-IR fragment into the vector pBI-G and checked the integrity of the cloned fragment by restriction enzyme analyses (data not shown). Clones that display low background and high inducibility by doxycycline will then be cotransfected with the IGF-IR response plasmid with pTK-Hyg to allow selection with hyrgomycin. We will screen for clones with low background and high doxycycline dependent induction of the antisense IGF-IR.

SCID Mouse Model:

At the time of the grant application when the human-peripheral blood lymphocyte-severe combined immune deficient (hu-PBL-SCID) mouse model was proposed, it was considered to be an excellent model for studying human tumor biology (Mueller and Reisfeld, 1991; Cowing and Gilmore, 1992; Duchosal et al., 1992). Indeed, in collaboration with Dr. Joseph Ilan's laboratory we had observed that SCID mice reconstituted with human PBLs and injected with parental human glioblastoma cells (HTB15) developed tumors that were significantly smaller than tumors from unreconstituted (control) SCID mice (unpublished observations). This data suggested that an allogeneic response had occurred against the tumor cells in the reconstituted mice which indicated that the human immune system in the SCID mice was functional. However, a recent report (Tary-Lehmann et al., 1995) provided additional insights into the functionality of the human immune system in the reconstituted SCID mouse. It appears that following PBL injection the transferred cells are functional for the first 2-3 weeks and that human immune responses can be induced in hu-PBL-SCID mice. However, three weeks after grafting, human lymphocytes in the chimeras are nonfunctional. It is now apparent that the first generation of the hu-SCID-mouse is not useful for the evaluation of the peripheral human immune system (McCune, 1997). Therefore, in its original form, the model is suboptimal since it does not provide a stable, functional human immune system in a mouse. We are collaborating with Dr. Tary-Lehmann's laboratory in the development of a hu-PBL-SCID mouse model that can maintain a functional human immune system over a greater time span, and thereby be more suitable for long term experiments in tumor biology and the testing of treatment modalities targeted to human cells.

IGF-IR Antisense in Human Breast Cancer Cells, MD-MB-435S:

We have previously established that the antisense IGF-IR transfected MDA-MB-435S breast cancer cells have a reduced growth rate *in vitro*. In parallel to the decreased growth, we observed an increase in the number of non-adherent cells in the cultures

containing the antisense IGF-IR transfectants. A Trypan blue exclusion assay of these cells showed that the majority of the non-adherent cells were dead. Several cancers, including breast overexpress IGF-IR. Activation of this receptor in cancer cells may inappropriately retard apoptosis and thereby promote tumorigenesis. Therefore to determine whether the cell death of antisense IGF-IR transfectants was due to apoptosis, DNA fragmentation assays were performed. DNA laddering was apparent after 5 days in serum free medium in the C8 and C9 antisense transfected cell clones (Figures 1A and 1B, Lanes 4 and 6, respectively, Appendix I). In contrast, CMV (control) transfected cells (Figures 1A and 1B, Lanes 1 and 2, Appendix I) did not show DNA laddering under the same conditions. Our results indicate that IGF-IR inhibition by antisense in human breast cancer cells leads to apoptosis. This could be one of the mechanisms that inhibits the tumor growth of the antisense IGF-IR transfected cells.

The role of immunosurveillance in breast cancer is as yet undefined. Both cellular and humoral componenets of the immune system are thought to be involved in the immunologic response to neoplasia. The humoral aspects are difficult to study using human cell lines or human grafts. However, some aspects of cell mediated immunity can be examined *in vivo* by using the diverse immune compromised animal models that are available.

We have previously reported (Ilan et al., 1997) that human breast cancer cells, MDA-MB-435S transfected with antisense to IGF-IR exhibit a significant delay in tumor formation and dramatically suppressed tumor growth when injected into nude mice. This animal model is often used to assess tumor take and tumor growth of human cells, however nude mice usually do not develop spontaneous metastasses and are not suitable for all types of tumors. Nude mice are deficient in T cells, but could mount an immune response against tumor cells from other components in their immune system, namely B, NK, LAK and macrophages. SCID mice are more severely immune deficient lacking both T and B cells while SCID beige mice are the most immune deficient lacking T, B and NK cells. These animal models provide an *in vivo* system for assessing the role of the immune system in tumorigenesis and metastases. Therefore, to determine how the mouse immune system affects tumorigenesis and metastases of MDA-MB-435S human breast cancer cells, a bioassay using these animal models was carried out. In addition, the tumorigenic and metastatic behavior of the MDA-MB-435S cells transfected with antisense to IGF-IR was evaluated in these animal models.

Three types of mice, nude, SCID and SCID-beige were injected subcutaneously in the left scapular region with 2 X 10^6 parental, control transfected (CMV) or antisense IGF-IR transfected MDA-MB-435S cells. The tumor growth was monitored and the mice were sacrificed at the end of week 11. The tumors were excised and weighed. The lungs were also removed, fixed in Bouins solution and examined under a dissecting microscope for metastases.

We found that all groups of mice had similar tumor take rates, however the SCID beige animals displayed a greater incidence of lung metastases with parental or control transfected cells (Table 1, Appendix I) than either nude or SCID mice. The tumors were

significantly smaller in all mice injected with the antisense IGF-IR transfected cell clones (C8 and C9) (Figure 2, Appendix I) than in mice injected with parental or control transfected cells. In addition, there was no incidence of lung metastases in any of the mice receiving the antisense IGF-IR transfected cell clones (Table 1, Appendix I).

Our results indicate that the SCID biege mouse may be a more suitable model for studying the metastatic potential of the human breast cancer cell line, MDA-MB-435S. Other groups (Lee and Welch, 1997: Price et al., 1990) have used the nude mouse to study lung metastases from MDA-MB-435S breast cancer cells, however they have reported that it takes 18-30 weeks for the cells to metastatize to lung. Our experiment showed that the incidence of lung metastases is 100% in SCID beige mice after 11 weeks indicating that this animal model may be more suitable for studying the metastatic potential of these human breast cancer cells and for evaluating experimental antimetastatic therapies. Indeed, we found that SCID beige mice injected with antisense IGF-IR transfected MDA-MB-435S cell clones did not have lung metastases at 11 weeks post injection which could ultimately prolong the lifespan of the animal. Additional studies are planned to determine how long animals injected with antisense IGF-IR MDA-MB-435S transfectants will survive and if pulmonary metastases are inhibited or reduced..

The Role of the Immune System following Treatment of Tumor Cells with Antisense IGF-IR or Antisense TGF-β:

We (Trojan et al., 1993; Trojan et al., 1996) have previously shown that suppression of IGF-I by an antisense gene targeting approach inhibits tumor growth of C6 glioblastoma cells by evoking an immune response. A recent report (Resnicoff et al., 1996) has demonstrated that expression of antisense IGF-IR in C6 glioblastoma cells inhibits tumorigenicity by induction of a cellular immune response as well as by additional undefined mechanisms. Therefore, we carried out a series of preliminary experiments on the murine breast cancer cell line, EMT6, to determine whether the immune system plays a role in the inhibition of tumorigenesis that occurs when these cells are transfected with either antisense to IGF-IR or antisense to TGF-β.

Syngeneic Balb/C mice were injected intraperitoneal (i.p.) with 0.5×10^5 parental, control transfected, antisense IGF-IR transfected cell clones or antisense TGF-β transfected EMT6 cells. The animals were sacrificed after 12 days and the peritoneal cavities examined for tumor and ascites formation. All of the mice injected with the parental EMT6 cells or the control (CMV) transfected EMT6 cells developed massive ascites with a high tumor burden (Table 2, Appendix I). On the other hand, two of the mice injected with the antisense IGF-IR cell clones (H and J) remained tumor free while the remaining 7 mice from the H and J cell clones developed a small amount of ascites with a minimal tumor burden (Table 2, Appendix I). In the group of mice injected with the EMT6 cells containing antisense to TGF-β, two of the animals remained tumor free while the three remaining mice developed a small amount of ascites with minimal tumor burden, similar to that seen in mice injected with the antisense IGF-IR transfected cells (Table 2, Appendix I).

We carried out experiments to determine the cytokine profile of EMT6 cells and whether this profile was altered in EMT6 breast cancer cells transfected with vectors producing antisense to IGF-IR or TGF-β. In these initial studies we focused on interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5) and interferon gamma (IFNγ) since IL-2 and IFNγ are involved in tumor rejection and IL-4 has been implicated in immune tolerance in tumor cells.

In order to establish a baseline cytokine profile for EMT6 cells *in vitro*, the parental, CMV transfected, antisense IGF-IR cell clones and the antisense TGF-β cells were assessed by ELISA-spot assays (Figure 3, Appendix I). The entire panel of EMT6 tumor cells tested positive for IL-2 and negative for IL-4 (Figure 4, Appendix I). In addition, none of these cultured cells produced IL-5 or IFNγ (data not shown). We then performed a cytokine profile on spleen cells obtained from mice injected with PBS and challenged these cells with irradiated parental EMT6 cells, CMV (control) transfected, antisense IGF-IR transfected or antisense TGF-β transfected cells (Table 3A, Appendix I). The cytokine profile for this panel of cells showed a production of IL-4 in all cells tested (Figure 5, Appendix I). Furthermore, these cells did not produce IL-2, IL-5 or IFNγ (data not shown).

In the next experiment, spleen cells from the animals were challenged with tumor cells from the same cultures as the cells used for injection into the mice (Table 3B, Appendix I). An up regulation of IL-4 and IFNγ occurred when spleen cells from animals injected with parental EMT6 cells were challenged with cultured parental EMT6 cells (Figure 6, Appendix I) or when animals injected with the CMV (control) transfected cells were challenged with cultured parental EMT6 or CMV transfected EMT6 cells (Figure 7, Appendix I). A similar increase in production of IL-4 and IFNγ was seen in the spleen cells of animals injected with antisense IGF-IR and challenged with either cultured antisense IGF-IR EMT6 cells or parental EMT6 cells (Figure 8, Appendix I). A small up regulation in IL-4 and IFNγ was seen when the spleen cells of animals injected with the antisense TGF-β transfected EMT6 cells were challenged with cultured antisense TGF-β transfected EMT6 cells or parental EMT6 cells (Figure 9, Appendix I).

These initial experiments have shown that EMT6 cells produce IL-2 and that transfection with antisense to IGF-IR or TGF-β does not alter the status of this cytokine within the cell under culture conditions. We demonstrated that naive spleen cells secrete IL-4 and that when challenged with a panel of EMT6 cells including parental, control transfected, antisense IGF-IR transfected or antisense TGF-β transfected cells, the IL-4 production is unchanged. Spleen cells from animals that were challenged with the same type of cultured cells used for the initial injection or challenged with parental EMT6 cells displayed up regulation of IL-4 and IFNγ. This series of experiments provide information regarding the production of IL-2, IL-4, IL-5 and IFNγ by EMT6 breast cancer cells. Further experiments are needed to determine the profile of other cytokines such as IL-10 and IL-12 which could be involved in evoking an immune response.

To determine the cytotoxic potential of stimulated lymphocytes towards EMT6 cells, antisense TGF-β transfected EMT6 cells and antisense IGF-IR transfected EMT6 cells, we used a standard ^{51}Cr release assay. We observed that the EMT6 tumor cells transfected with antisense TGF-β generated a strong cytotoxic T cell response when compared to the response of parental EMT6 cells (Figure 10, Appendix I). On the other hand neither the control transfected EMT6 cells nor the antisense IGF-IR transfected EMT6 cells exhibited a significant cytotoxic response (data not shown). This data suggests that the antisense TGF-β transfected cells induced a cellular immune response towards the EMT6 cells.

Another potential mechanism for tumor suppression could be through apoptosis. Therefore, we performed a pilot experiment to determine whether the EMT6 cells transfected with antisense IGF-IR or antisense TGF-β were undergoing apoptosis *in vivo*. Cells from tumors in syngeneic Balb/C mice were stained with propidium iodide followed by analysis with flow cytometry (FACS). The antisense TGF-β transfected EMT6 cells displayed markedly reduced apoptosis when compared to CMV transfected (control) cells (Table 4, Appendix I). On the other hand, antisense IGF-IR transfected EMT6 cells did not appear to differ in the percentage of cells undergoing apoptosis from CMV (control) transfected EMT6 cells (Table 4, Appendix I). This preliminary data suggests that tumor suppression using antisense IGF-IR or antisense TGF-β may occur through different routes and/or mechanisms.

Conclusions:

The long term goal of this study is to develop a gene therapy treatment to cure human breast cancer. We have carried out studies on a highly metastatic human breast cancer cell line MDA-MD-435S that was derived from the parental MDA-MB-435 cell line. We were able to consistently obtain tumors when 2 X 10^6 cells were injected *s.c.* into 6 week-old female nude mice. We have transfected the MDA-MB-435S cells with antisense IGF-IR constructs and have established eleven stable clones that have a significantly reduced expression of IGF-IR transcripts compared to control transfected cells. We found that a 40% inhibiton of IGF-IR expression is sufficient to reduce growth rate and to prevent the cells from forming anchorage independent colonies in soft agar assay.

Apoptosis is one mechanism that could contribute to the reduction in tumorigenicity of the antisense IGF-IR transfected cells. Recent reports have suggested that overexpression of IGF-IR can inhibit apoptosis in C6 rat glioblastoma cells (Resnicoff et al.,1995), Balb/c 3T3 cells (Sell et al.,1995) and a neuroblastoma cell line (Singleton et al.,1996). Furthermore, inhibition of IGF-IR in a Ewing's sarcoma/peripheral neuoectodermal tumor cell line (Scotlandi et al.,1996) induces apoptosis *in vitro*. We observed that apoptosis was enhanced in MDA-MB-435S human breast cancer cells expressing antisense to IGF-IR. Therefore, inhibition of endogenous IGF-IR by the anitsense approach induces an apoptotic response similar to that reported for other types of tumor cells. The significant delay in onset of tumor growth and the dramatic reduction in the tumorgenicity of antisense-IGF-IR transfected MDA-MB-435S cells in nude mice appears to be at least in part due to apoptosis.

We could not rule out the involvement of the remnant immune components which exist in nude mice, eg. natural killer cells and/or machrophages. Therefore, to determine whether the nude mouse immune system contributed to the suppression of tumor growth in the antisense IGF-IR transfected human breast cancer cells, we carried out a bioassay with immunecompromised animal models (nude, scid and scid beige mice). There was a similar reduction in tumor size and a delay in onset of tumor formation in all of the mice injected with the MDA-MB-355S carrying the antisense IGF-IR construct. In addition, there was a complete absence of pulmonary metastasis in all of the mice injected with the antisense IGF-IR transfected breast cancer cells. On the other hand, while all of the animals injected with parental or control transfected MDA-MB-435S cells developed large tumors, only the scid beige mice consistantly exhibited pulmonary metastases. The scid beige mouse, the most immune compromised of the available mouse models, appears to be highly suitable for studying the metastatic behavior of the human breast cancer cells since the rudimentary immune system components in the nude and scid mice could prevent or delay the onset of pulmonary metastases.

Further experiments are planned to study the role of IFG-IR in the metastases of breast cancer cells. We plan to do a series of survival experiments using scid beige mice to determine whether treatment of the MDA-MB-435S cells with antisense to IGF-IR can

prevent pulmonary metastases. The major cause of death in breast cancer is metastases that are resistant to conventional therapies. The etiology of metastases is a result of the interaction of metastatic cells with factors provided by the host environment. Indeed, ninety percent of deaths in breast cancer patients occur when the tumor has metastasized to bone (Yoneda et al., 1994). Breast cancer cells may prefer bone to other organs because of the abundance of insulin-like growth factors in the microenvironment. Breast cancer cells express the IGF-IR, therefore, they have the ability to respond to bone derived IGFs. To investigate whether bone derived IGFs contribute to the osteolytic bone metastases that occur in breast cancer, we will assess the effect of blocking the IGF-IR on human breast tumor cells that are implanted in the calvaria of nude and scid beige mice. We have previously used an animal model to demonstrate that rat prostate cancer cells transfected with the antisense IGF-IR construct and placed over the abraded calvaria did not invade into the brain parenchyma, whereas the control transfected cells formed a large tumor mass with extensive invasion into the brain parenchyma (Burfeind et al., 1996).

We have expanded our studies on the mouse breast cancer cell line EMT6 to determine whether the immune system plays a role in the inhibition of tumorigenesis that occurs when antisense IGF-IR or antisense TGF-β transfected cells are injected into nude mice. We have previously reported that tumor inhibition occurred in nude and immunocompetent Balb/c mice injected with EMT6 cells transfected with antisense-IGFIR. However, when we injected EMT6 cells transfected with the antisense TGF-β construct into nude and syngeneic animals, tumor inhibition occurred only in the syngeneic Balb/C mice. This experiment suggested that an intact immune system is essential for tumor inhibition with antisense TGF-β treatment while other undefined mechanisms are involved in the suppression of tumorigenesis by the antisense IGF-IR approach. Several groups have shown that injection of tumor cells transfected with antisense IGF-I (Trojan et al.,1993) or with antisense IGF-IR (Reniscoff et al.,1994) prevents subsequent wild-type tumorigenesis and induces regression of established tumors. More recently, Reniscoff et al. (1996) have demonstrated that a cellular immune response is evoked in syngeneic rats following the injection of glioblastoma cells expressing antisense IGF-IR.

We have isolated and characterized several antisense IGF-IR transfected EMT6 clones as well as several antisense TGF-β transfected cell clones. We carried out *in vitro* assays to test for cell proliferation and an altered phenotype in the transfected cell clones. There was no difference in rate of cell growth and the ability of the cells to form colonies in soft agar between clones carrying antisense IGF-IR, antisense TGF-β and control (CMV) transfected cells. On the other hand, the antisense IGF-IR transfected and the antisense TGF-β transfected EMT6 cell clones displayed significant inhibition of tumorigenesis in immunocompetent animals. Therefore, studies have been initiated to determine what mechanisms, i.e. immune functions, apoptosis or possibly other undefined factors, may be involved in the tumor suppression. We performed pilot experiments to determine the cytokine profile of EMT6 cells and EMT6 cell clones carrying the antisense constructs. We found that all tumor cells produce interleukin-2 in culture, whereas the tumor cells produce interleukin-4 *in vivo*. These experiments provide information regarding the production of some cytokines by murine tumor cells, however additional

studies are needed to determine whether other cytokines such as IL-10 and IL-12 are produced by breast cancer cells carrying antisense to IGF-IR or TGF-β. We have obtained pilot data that indicates that apoptosis is involved in tumor suppression of antisense TGF-β transfected EMT6 cells. Our results on the EMT6 cells suggest that reduced tumorigenesis is occuring through both immune and apoptotic routes as well as other as yet undefined mechanisms.

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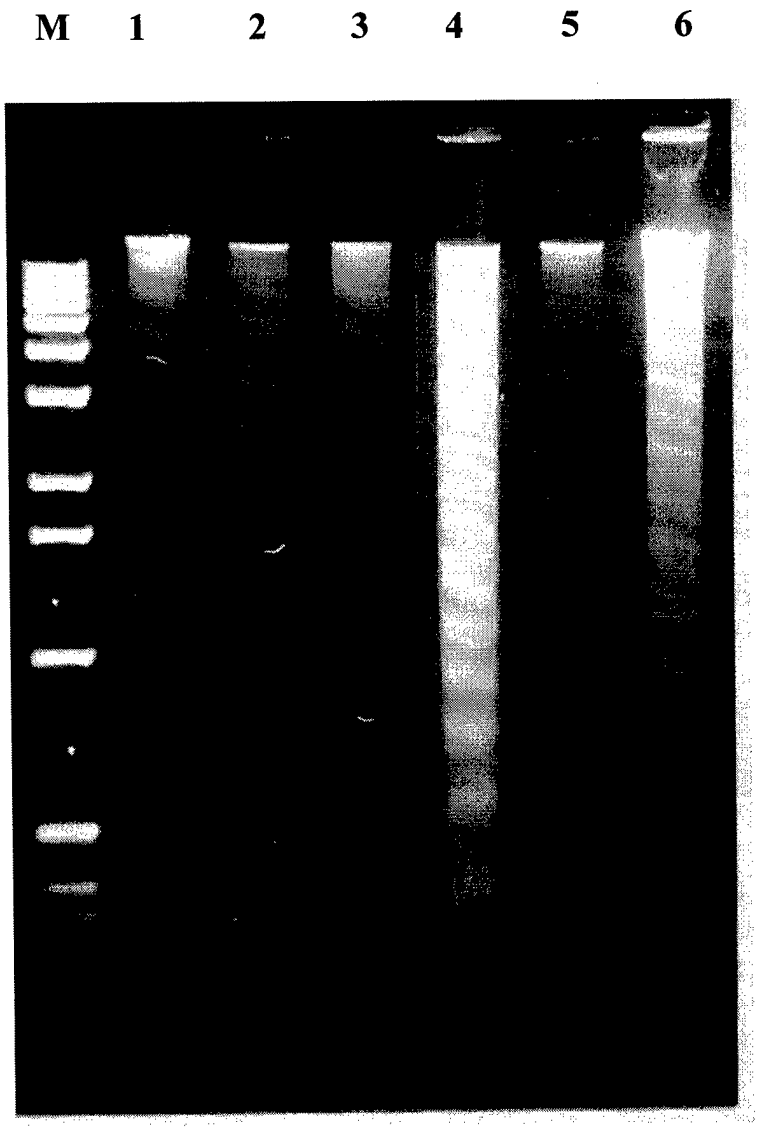

Figure 1A. Apoptosis of non-adherent antisense IGF-IR transfected MDA-MB-435S cell clones C8 and C9. 2 X 10^6 of each cell line was seeded in 15 ml DMEM + 10 % FCS. The culture medium was replaced with serum free medium the next day. Non-adherent cells were harvested at indicated time points and DNA extracted using Promega Genomic DNA isolation kit according to the manufacturer's protocol.
DNA fragmentation analysis of the non adherent cells was performed by agarose gel (1.5%) electrophoresis and ethidium bromide staining.
Lane M: Molecular weight markers (1 kb ladder). Lanes 1, 2: CMV control transfected MDA-MB-435S cells. Lanes 3, 4: antisense IGF-IR transfected MDA-MB-435S Clone 8, Lanes 5, 6: antisense IGF-IR transfected MDA-MB-435S Clone 9. Lanes 1,3,5: Day 2 of serum withdrawal. Lanes 2,4,6: Day 5 of serum withdrawal.

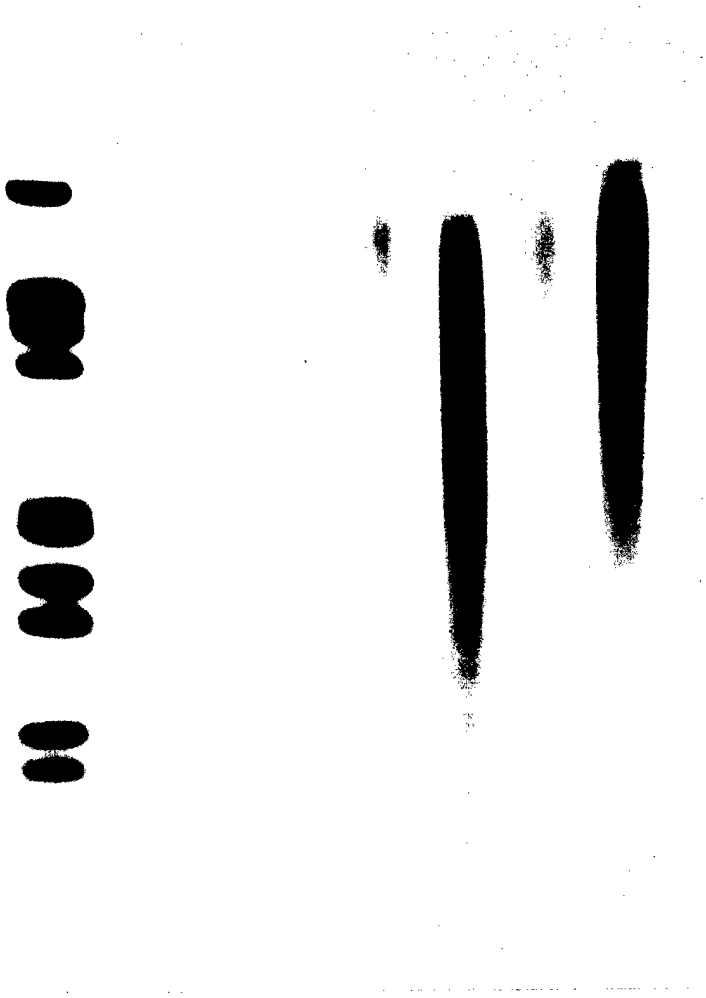

Figure 1B. Induction of apoptosis in adherent and non-adherent antisense IGF-IR transfected MDA-MB-435S cells clones C8 and C9. 1 X 10^6 cells were seeded in 15ml DMEM + 10% FCS. The medium was replaced with serum free DMEM the next day. Adherent and non-adherent cells were combined and harvested at the indicated time points of serum withdrawal. DNA was isolated using Promega Genomic DNA isolation kit and 4μg of each cell line was used for labeling of internucleosomal fragments with ^{32}P-ATP using *Taq* Polymerase (Eldadah et al., 1996). The labeled DNA was electrophoresed on a 1.5% agarose gel and visualized by autoradiography. Lane M: Molecular marker λ*Eco*RI/*Hind*III ladder. Lanes 1,2 CMV control transfected MDA-MB-345S. Lanes 3, 4: antisense IGF-IR transfected MDA-MB-435S Clone 8, Lanes 5, 6: antisense IGF-IR transfected MDA-MB-435S Clone 9. Lanes 1,3,5: Day 3 serum withdrawal. Lanes 2,4,6: Day 5 serum withdrawal.

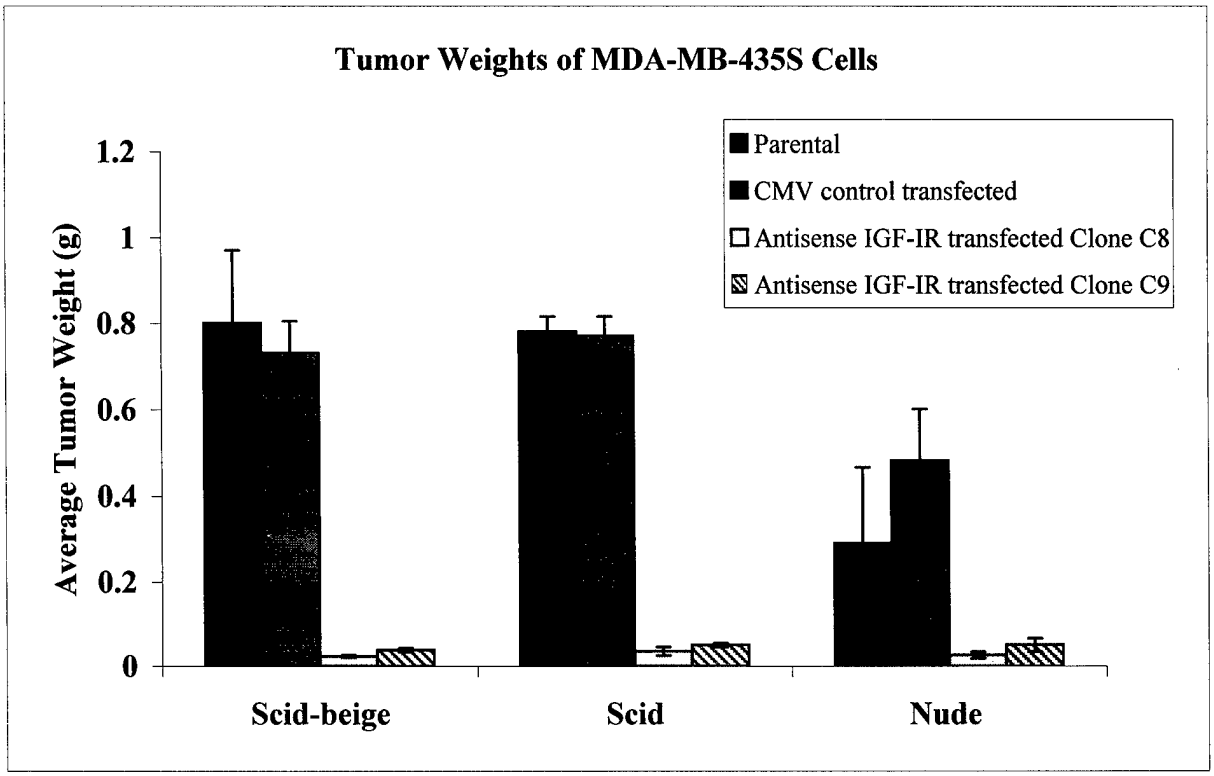

Figure 2. Suppression of tumorigenesis by antisense-IGF-IR transfected MDA-MB-435S cells in scid-beige, scid and nude mice. The animals were injected subcutaneously with 2 X 10^6 cells and sacrificed 11 weeks later. Tumors were excised and weighed. Data are presented as mean + SE.

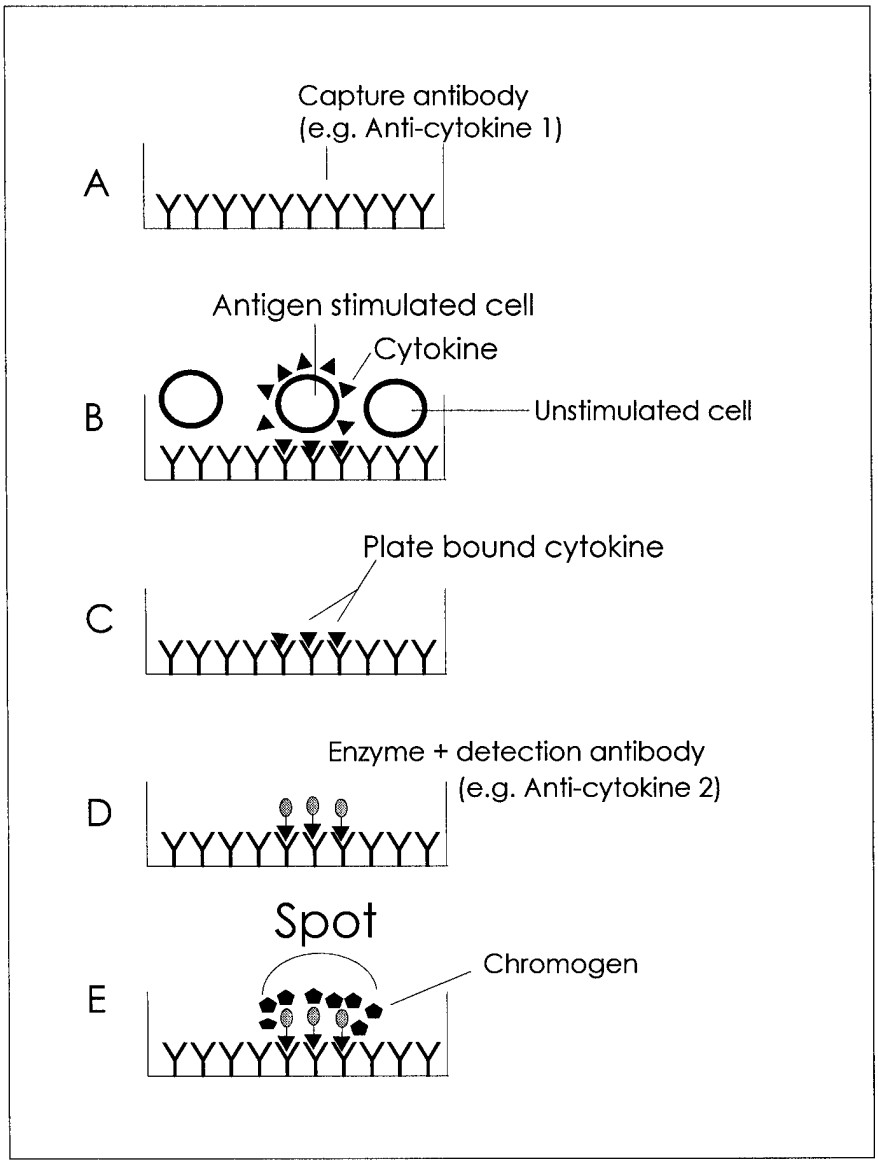

Figure 3. Schematic representation of the ELISA-spot assay.
(A) Wells of a 96 well microtiter plate are covered by a specialized membrane (Athersys Inc.,Cleveland, Ohio). Wells are coated with "capture" antibody to a cytokine, e.g. anti-IFNγ (anti-cytokine 1), and excess antibody were washed off. (B) The cell samples, e.g. spleen cells, were seeded at 1 X 10^6 cells/well. An experimental well containing the test antigen is depicted. Control wells contain an irrelevant antigen or medium alone. (C) After an incubation period of 12-48 hour at 37°C in a CO_2 incubator, and the cytokine released by each cell is trapped on the antibody coat. The cells are then washed off. (D) and (E) The presence of cytokine secreting cells is revealed when an enzyme labeled second anti-cytokine antibody, e.g. horse radish peroxidase labeled anti-IFNγ (anti-cytokine-2) is added. The cytokine released by each cell makes a distinct spot in this assay and is visualized by addition of chromogenic substrate.

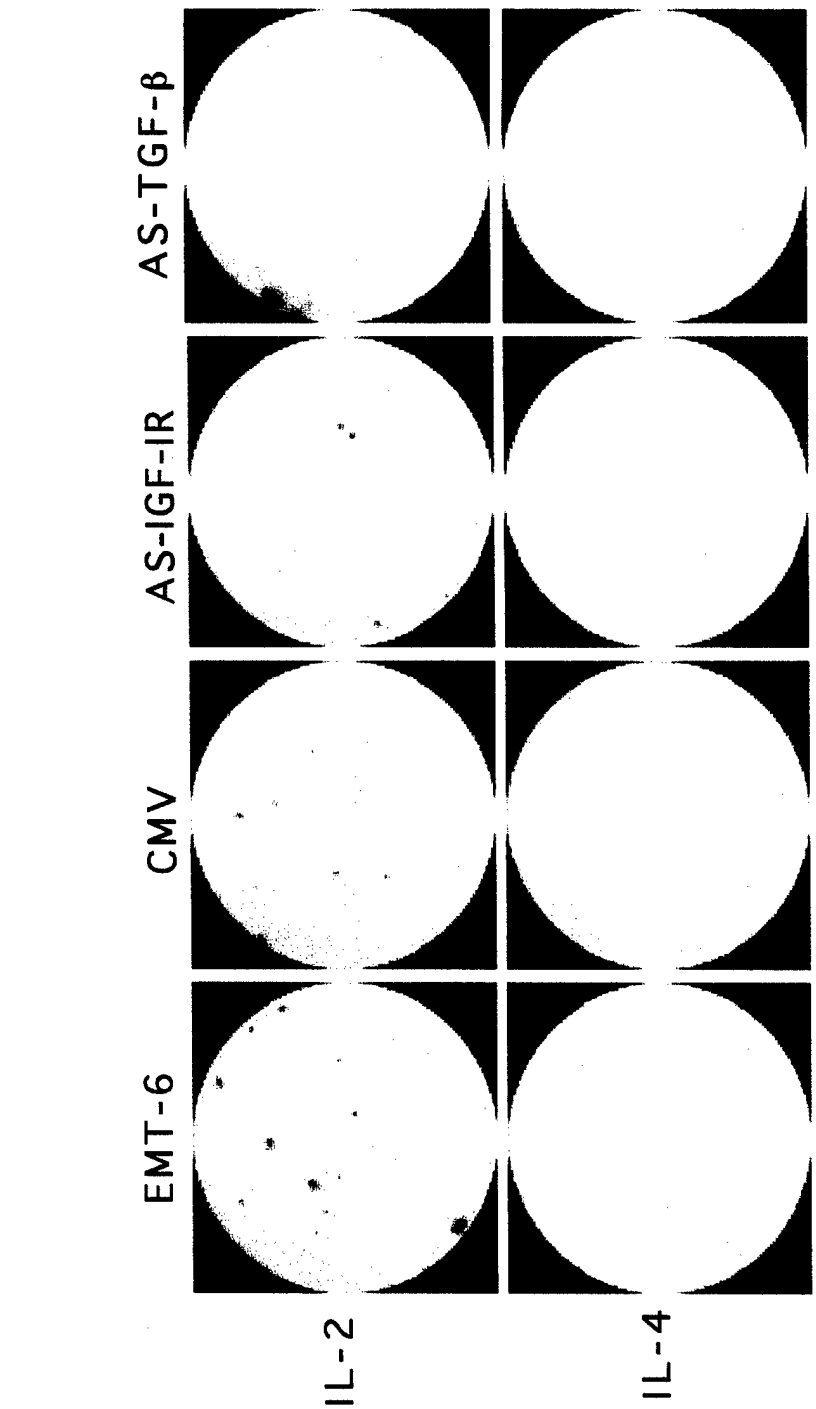

Figure 4. IL-2 and IL-4 production by parental, CMV control transfected, antisense IGF-IR transfected and antisense TGFβ transfected EMT6 cells in culture using ELISA spot assay.

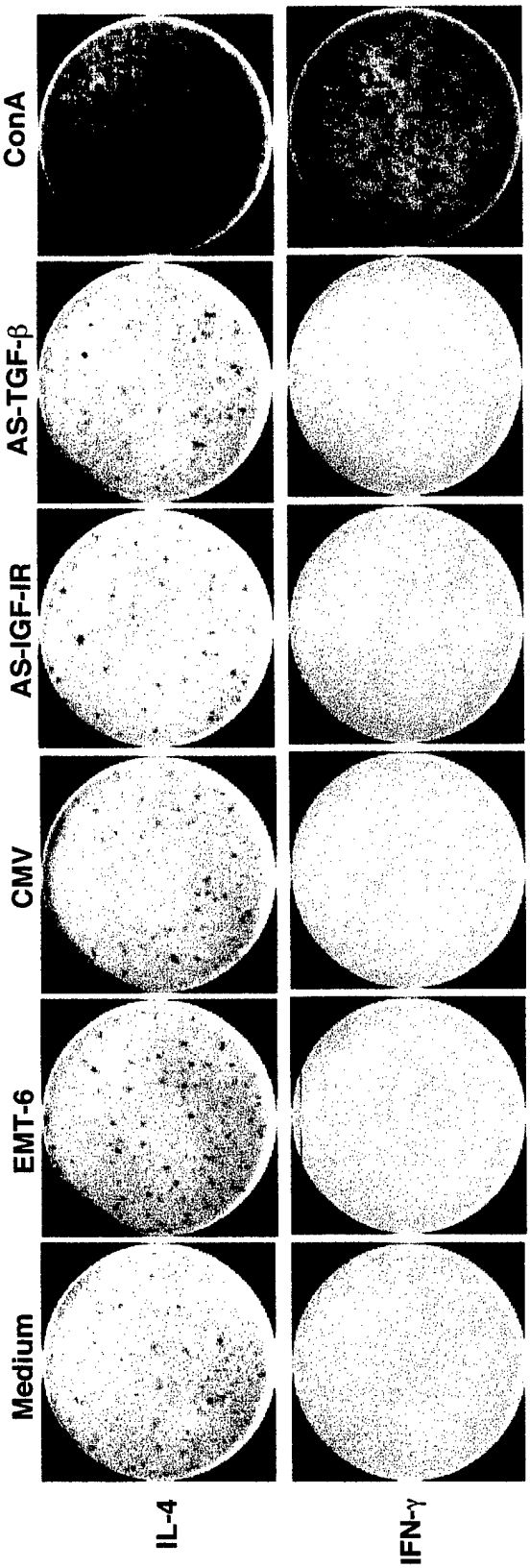

Figure 5. ELISA spot assay depicting IL-4 and IFNγ production in spleen cells obtained from a mouse injected *i.p.* with PBS. The spleen cells were challenged with irradiated cultured parental, CMV control transfected, antisense IGF-IR transfected, antisense TGFβ transfected EMT6 cells, medium (negative control) or concanavalin A (ConA) (positive control).

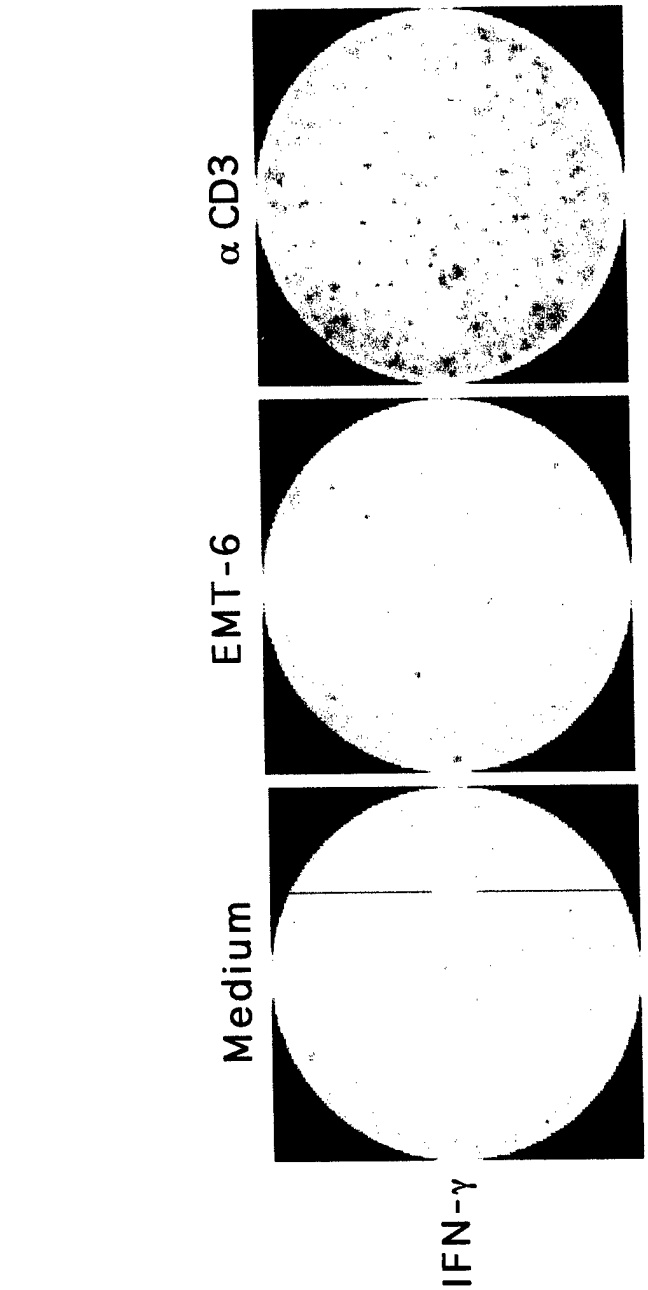

Figure 6. ELISA spot assay depicting IL-4 and IFNγ production in spleen cells obtained from a mouse injected *i.p.* with parental EMT6 cells (0.5×10^5). The spleen cells were challenged with irradiated cultured parental EMT6 cells, medium (negative control) or ConA (positive control).

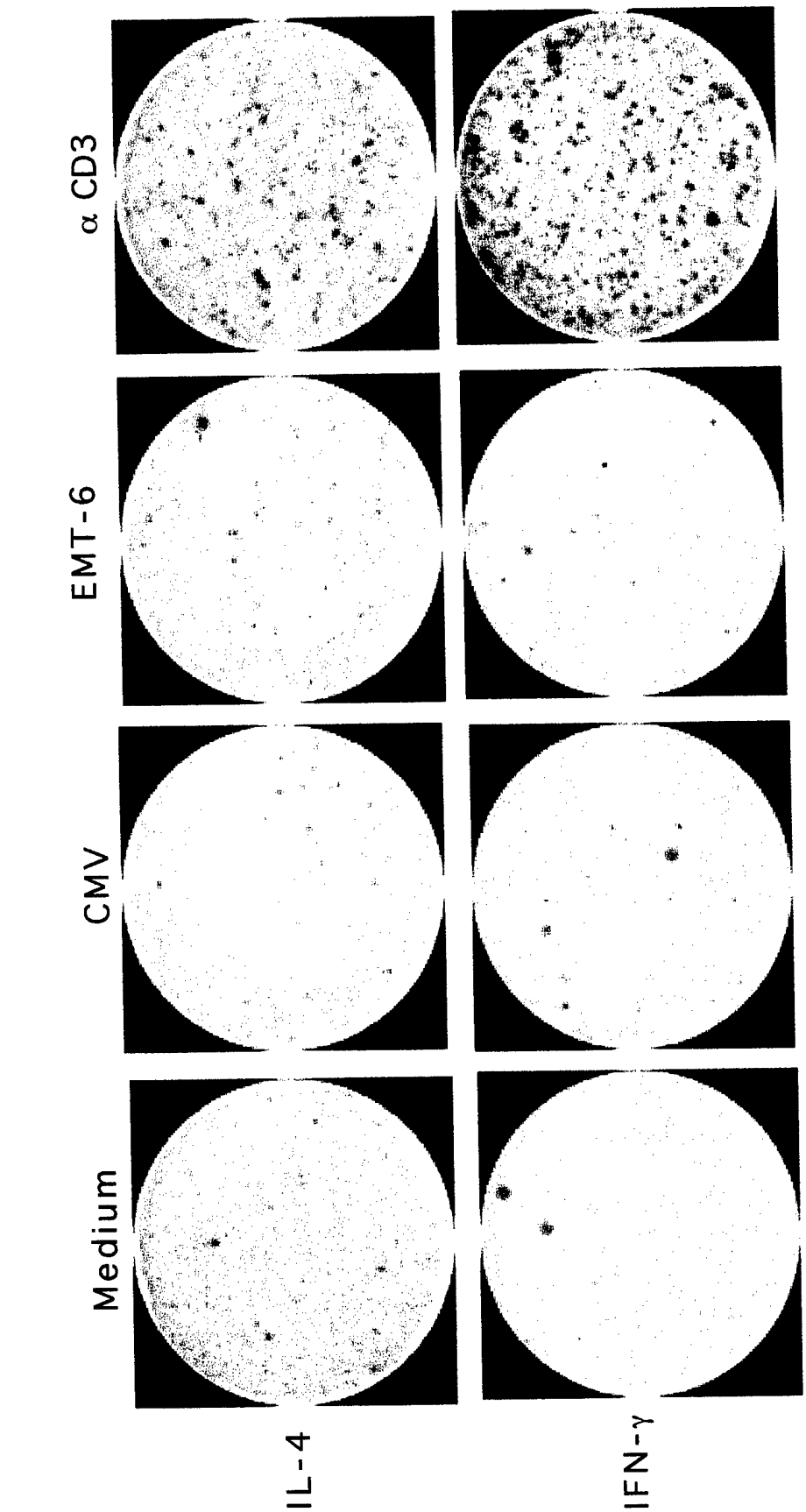

Figure7. ELISA spot assay depicting IL-4 and IFNγ production in spleen cells obtained from a mouse injected *i.p.* with CMV control transfected EMT6 cells (0.5×10^5). The spleen cells were challenged with irradiated cultured parental or CMV control transfected EMT6 cells, as well as medium (negative control) or ConA (positive control).

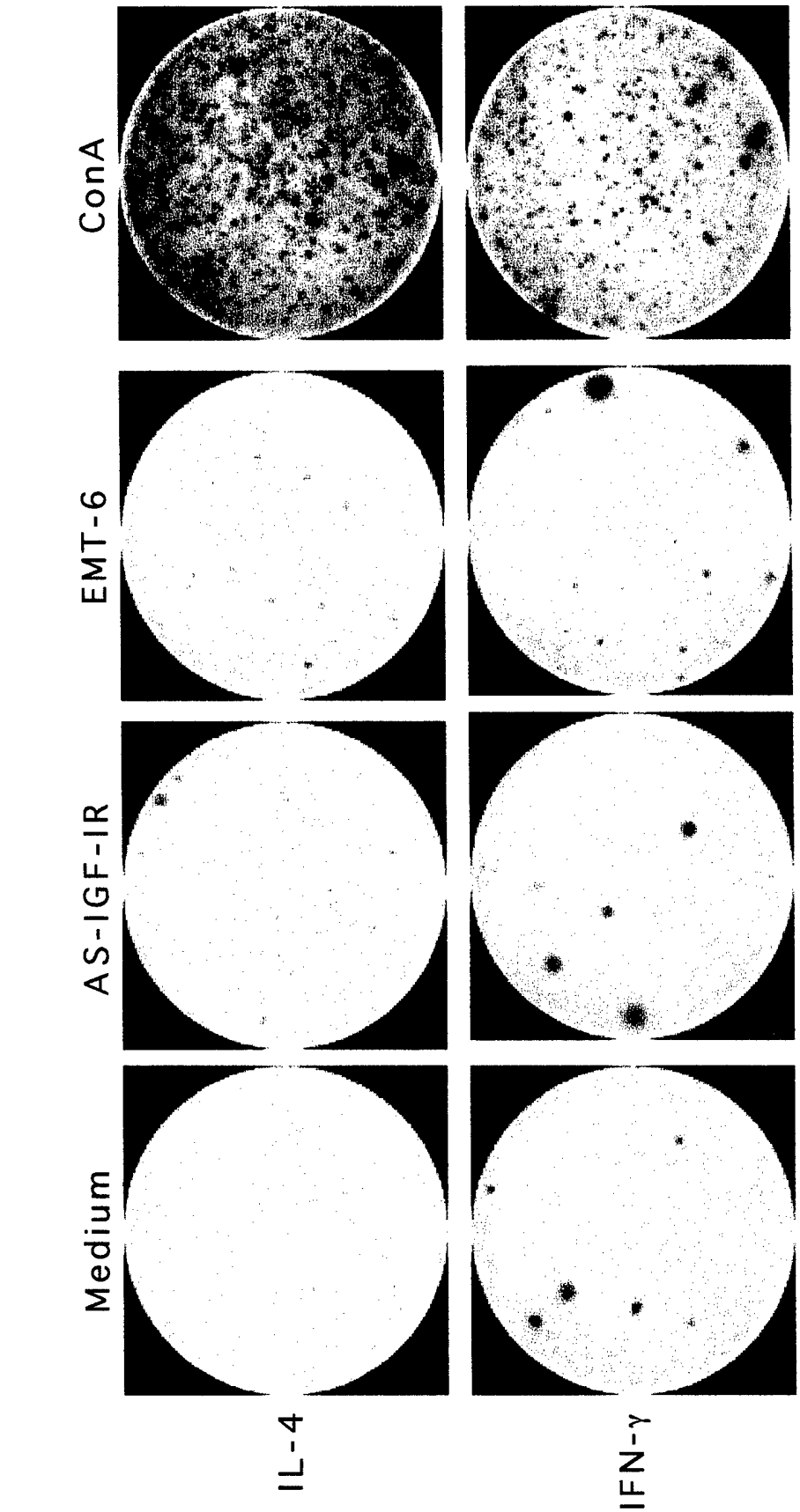

Figure 8. ELISA spot assay depicting IL-4 and IFNγ production in spleen cells obtained from a mouse injected *i.p.* with antisense IGF-IR transfected EMT6 cells (0.5×10^5). The spleen cells were challenged with irradiated cultured parental or antisense IGF-IR transfected EMT6 cells, as well as with medium (negative control) or ConA (positive control).

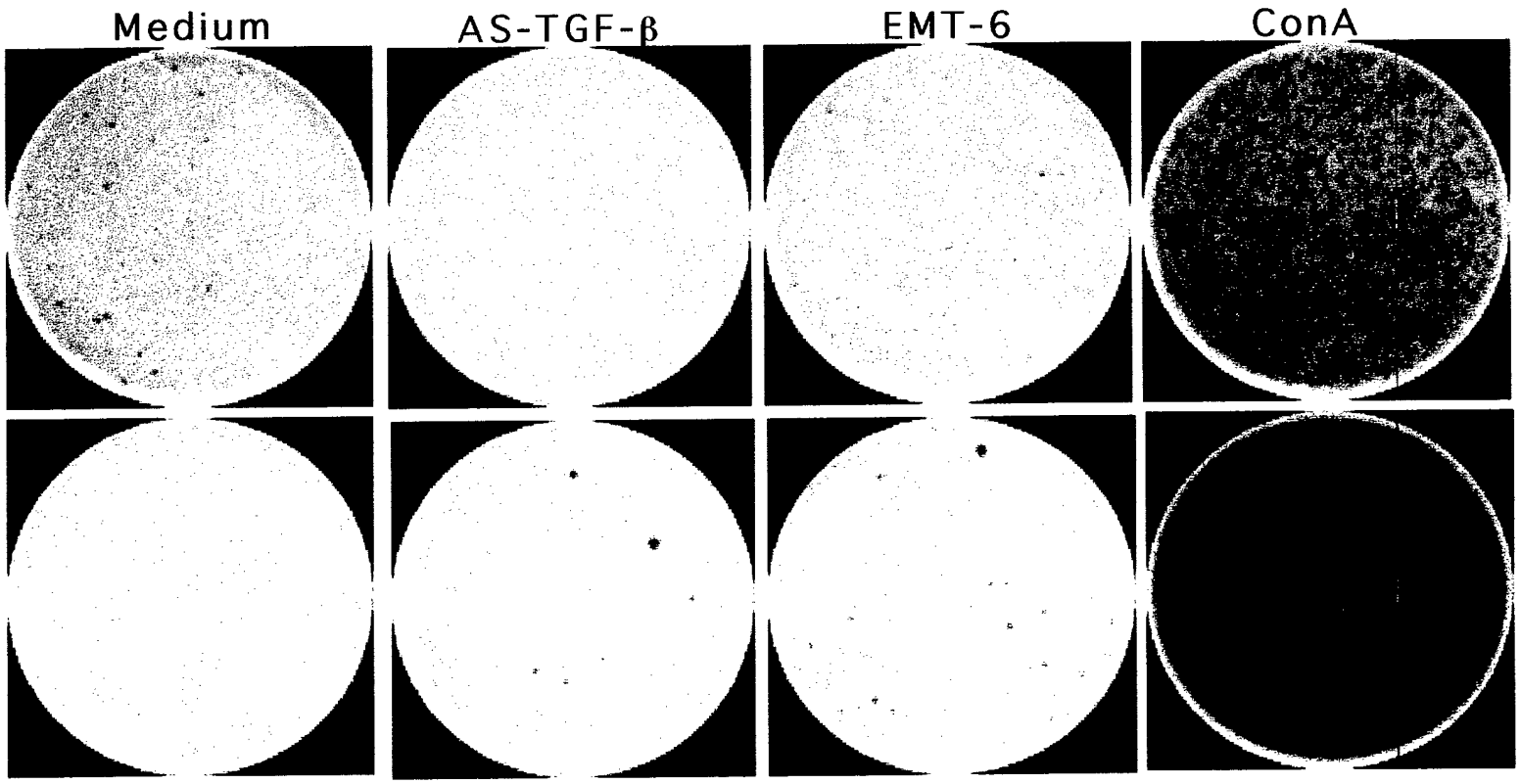

Figure 9. ELISA spot assay depicting IL-4 and IFNγ production in spleen cells obtained from a mouse injected *i.p.* with antisense TGFβ transfected EMT6 cells (0.5 X 10^5). The spleen cells were challenged with irradiated cultured parental or antisense TGFβ transfected EMT6 cells, as well as with medium (negative control) or ConA (positive control).

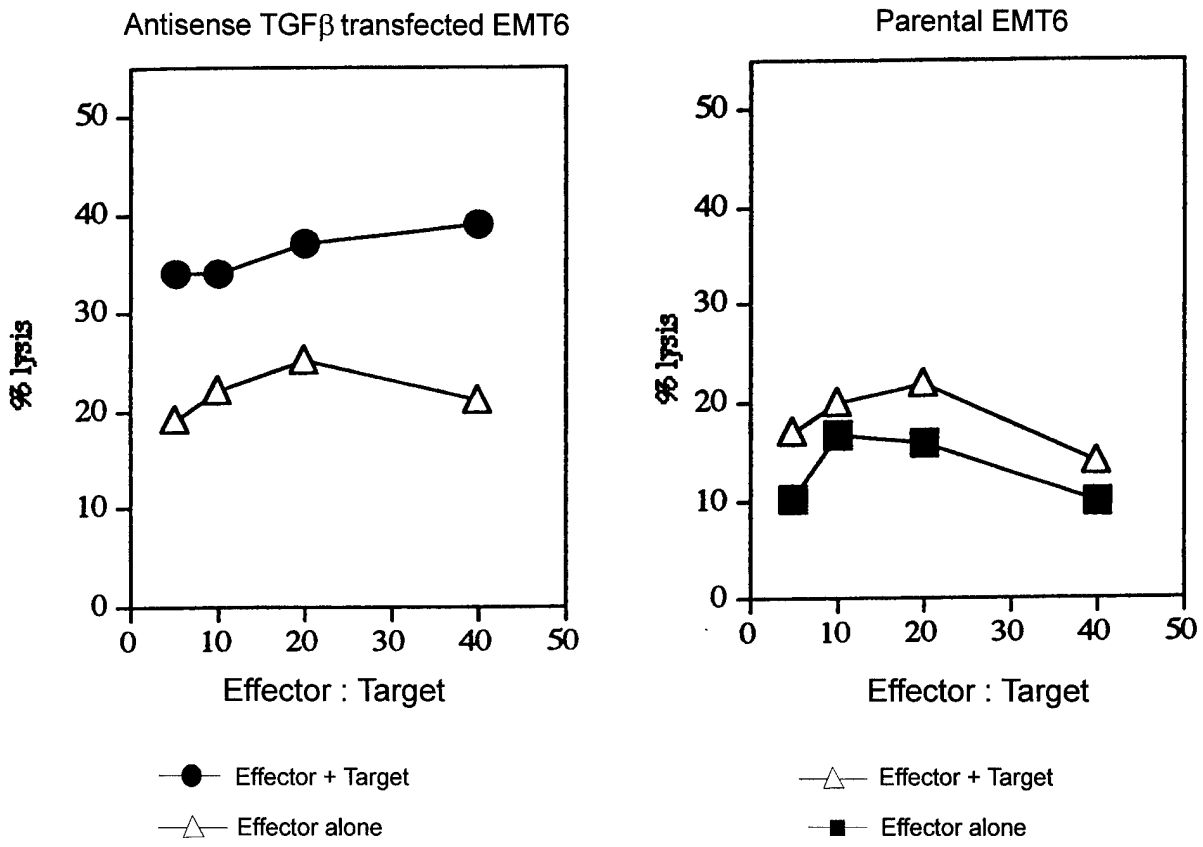

Figure 10. Cytotoxic response of splenocytes from mice injected with parental or antisense TGFβ transfected EMT6 cells using a standard ^{51}Cr-release assay. Splenocytes (4 x 10^6) from mice injected with parental or antisense TGFβ transfected EMT6 cells were used as effector cells against the EMT6 parental target cells at effector: target ratios of 5, 10 20 and 40. Wells containing only target cells with either culture medium or 5% Triton X served to determine spontaneous and maximal ^{51}Cr release respectively. Percent-specific lysis of cells releasing ^{51}Cr was calculated from the counts released into the supernatant after a 4-hour incubation period using the formula

Cytotoxicity (%) =

$$\frac{\text{Experimental release (cpm)- spontaneous release (cpm)}}{\text{Maximal release (cpm) – spontaeous release (cpm)}} \times 100\%$$

TABLE 1. Incidence of lung metastases in 3 immunocompromised mouse models injected *s.c.* with parental, CMV control transfected or antisense IGF-IR transfected MDA-MB-435S cell clones (C8 or C9).

Cells injected	Mice with lung metastases		
	Scid-beige	Scid	Nude
Parental	5/5	1/5	1/5
CMV	4/4	1/5	0/5
C8	0/5	0/5	0/5
C9	0/3	0/5	0/5

TABLE 2. Tumor incidence in syngeneic Balb/c mice injected i.p. with parental, CMV control transfected, antisense IGF-IR transfected (Clones J and H) or antisense TGFβ transfected EMT-6 cells (0.5×10^5).

Cells injected	**Tumor-free mice**	**Mice with minimal tumor and ascites**	**Mice with massive tumor and ascites**
EMT-6 parental (n=6)	0	0	6
CMV control (n=6)	0	0	6
IGF-IRAS Clone J (n=3)	1	2	0
IGF-IRAS Clone H (n=6)	1	5	0
AS TGF-β (n=5)	2	3	0

Table 3A. Cytokine production in cultured parental, CMV control transfected, antisense IGF-IR transfected or antisense TGFβ transfected EMT6 cells determined by ELISA-spot assay.

Cell Line	Cytokine Product
EMT6 parental	IL2
CMV vector control transfected EMT6	IL-2
Antisense IGF-IR transfected EMT6, Clone H	IL-2
Antisense IGF-IR transfected EMT6, Clone J	IL-2
Antisense TGF-β transfected EMT6	IL-2

Table 3B. Cytokine production by spleen cells challenged with cultured irradiated parental, CMV control transfected, antisense IGF-IR transfected or antisense TGFβ transfected EMT6 cells.

Spleen cells obtained from Balb/c mice injected with	Irradiated EMT6 cells used for challenge	Cytokine Product
PBS	Parental EMT6	IL-4
	Antisense IGF-IR transfected EMT6	
	Antisense TGF-β transfected EMT6	
EMT6 parental	EMT6	IFN-γ and IL-4
CMV vector control transfected EMT6	EMT6	IFNγ and IL-4
	CMV vector control transfected EMT6	
Antisense IGF-IR transfected EMT6	EMT6	IFNγ and IL-4
	Antisense IGF-IR transfected EMT6	
Antisense TGFβ transfected EMT6	EMT6	IFNγ and IL-4
	Antisense TGFβ transfected EMT6	

Table 4. Percentage of apoptotic cells obtained from tumors in Balb/c mice injected with CMV control transfected, antisense IGF-IR transfected or antisense TGF-β transfected EMT6 cells.

Cells used for injection	**% apoptotic tumor cells**
CMV control transfected EMT6 (n=3)	35 ± 9
Antisense IGF-IR transfected EMT6 (n=3)	30 ± 18
Antisense TGFβ transfected EMT6 (n=3)	7 ± 3

% apoptotic cells expressed as mean ± S.E.

296-N. ANTISENSE RNA TO INSULIN-LIKE GROWTH FACTOR II OR THE TYPE I RECEPTOR AS WELL AS TRANSFORMING GROWTH FACTOR β SUPPRESSES TUMOR GROWTH OF HUMAN & MURINE BREAST CANCER

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Keywords (5): Antisense RNA, Type I IGF receptor, Transforming growth factor β, Tumorigenesis, Gene Therapy

EXTENDED ABSTRACT

Our proposal aimed to develop an approach that employs gene therapy for human breast tumors. We focused our efforts on targeting insulin-like growth factors (IGFs), the type I insulin-like growth factor receptor (IGF-IR) and transforming growth factor β (TGF-β) in human and murine breast cancer cells. Our work was based upon an antisense approach that we previously employed to treat rat glioblastoma (Trojan et al., 1992; Trojan et al., 1993) and mouse teratocarcinoma (Trojan et al., 1994) which utilized a construct expressing antisense to insulin-like growth factor I. Furthermore, we (Burfeind et al., 1996) and others (Resnicoff et al., 1994) have reported that antisense to IGF-IR can inhibit tumorigenesis of rat prostate cancer cells and rat glioblastoma.

The ultimate aim was to develop a database supporting the hypothesis that growth factors and their receptors are involved in the tumorigenicity of certain breast cancers. The objectives of our studies to date have been to develop model systems in which to analyze the effect of blocking the expression of IGFs, IGF-IR and TGF-β using antisense expression plasmids that we prepared. We have therefore carried out experiments on a highly metastatic human breast cancer cell line, MDA-MB-435S and a murine breast cancer cell line, EMT6.

The MDA-MB-435S human breast cancer cells express IGF type I receptor, therefore we transfected these cells with a plasmid carrying the IGF-IR antisense construct. We found that IGF-IR antisense inhibits the expression of endogenous IGF-IR in stably transfected MDA-MB-453S clones. Cell proliferation assays and soft agar assays of the antisense IGF-IR clones showed a decrease in growth rate and colony formation, respectively. The level of inhbition of IGF-IR in individual antisense transfected clones correlated with their growth rate and colony formation potential. Nude mice injected with MDA-MB-435S cells transfected with antisense IGF-IR constructs showed either a dramatic reduction or absence of tumor growth after 11 weeks. These data provide support for the role of IGF-IR in human breast tumorigenesis.

In parallel with the studies on the human cell line, we carried out a series of experiments on the murine breast cancer cell line, EMT6. The EMT6 cell line which has a syngeneic animal provided an *in vivo* system for analyzing the effect of blocking

expression of growth factors and their receptors as well as for assessing the host immune response. We found that EMT6 murine breast cancer cells express IGF-II, IGF-IR and TGF-β. Therefore, we carried out transfections of EMT6 cells with antisense constructs to block expression of these transcripts. EMT6 cells transfected with antisense to IGF-II displayed significant inhibition of tumor growth in syngeneic Balb/C mice. Following transfection of EMT6 cells with antisense to IGF-IR, we isolated several clones and selected clones that displayed high levels of IGF-IR antisense expression and low IGF-IR expression for further analysis. Cell proliferation assays carried out on the EMT6 cell clones transfected with the IGF-IR antisense construct showed a decrease in growth rate that correlated with the level of IGF-IR inhibition. We compared the *in vivo* growth characteristics of several of the antisense IGF-IR transfected EMT6 cell clones in nude mice and the syngeneic Balb/C mice. A dramatic inhibition of tumor growth in both nude and Balb/C mice was observed with all cell clones tested. In addition, we assessed whether plasminogen activator (PA) expression was altered in EMT6 cells transfected with antisense to IGF-IR. We found tissue type plasminogen activator (tPA) expression was significantly lower in the antisense IGF-IR cell clones than that of control transfected (vector minus the insert) cells. tPA is a protease that has been implicated in both tumorigenesis and metastasis, therefore inhibition of tPA via suppression of the IGF-IR could alter the tumor forming capabilities of the EMT6 cells. EMT6 cells also express TGF-βs which may play a role in mammary tumor formation as well as metastasis. When we injected EMT6 cells transfected with antisense to TGF-β2 into nude mice there was no difference in tumor size when compared to control transfected cells. However, when antisense TGF-β containing EMT6 cells were injected into syngeneic Balb/C mice there was a dramatic reduction in tumor size when compared to control transfected EMT6 cells indicating that a functional immune system is a prerequisite for tumor inhibition to occur with anitsense to TGF-β.

Our results show that the type I insulin-like growth factor receptor and transforming growth factor β play important roles in breast cancer tumorigenesis. In support of our studies, a recent report has shown that IGF-IR antisense oligonucleotide modified tumor cells can protect against peripheral disease and brain metastases in a rat breast cancer model (Wallenfriedmen et al., 1997). These studies taken together suggest that the antisense gene therapy approach could provide a basis for the development of new biologically targeted methods for treatment of human breast cancer.

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Appendix II

Suppression of insulin-like growth factor type I receptor by a triple-helix strategy inhibits IGF-I transcription and tumorigenic potential of rat C6 glioblastoma cells

(homopurine/triplex/nexin-I/tumorigenesis/gene therapy)

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ABSTRACT **Homopurine (AG) and homopyrimidine (CT) oligodeoxyribonucleotides predicted to form triple-helical (triplex) structures have been shown to specifically suppress gene expression when supplied to cultured cells. Here we present evidence that homopurine RNA (effector) sequences designed to form a triplex with a homopurine·homopyrimidine sequence 3′ to the termination codon of the insulin-like growth factor type I receptor (IGF-IR) structural gene can efficiently suppress IGF-IR gene transcription. Transfection vectors were constructed to drive transcription of either AG or CT variant triplex-forming strands. To increase the probability of obtaining stable transfectants with adequate expression of effector sequences, these were designed to be transcribed together with cDNA sequences conferring neomycin resistance as a fusion transcript. Rat C6 glioblastoma cells transfected with the AG variant showed dramatic reduction of IGF-IR transcripts compared with untransfected cells. The AG transfectants also exhibited marked down-regulation of the IGF-I, and an enhanced accumulation of serine protease inhibitor nexin-I mRNA. Similar changes in gene expression were observed following transfection of C6 cells with constructs transcribing antisense RNA to IGF-IR transcripts, but were not observed in C6 cells transfected with either the CT triplex variant or with vector lacking triplex-forming sequences. Moreover, C6 cells transfected with AG triplex variant displayed a dramatic inhibition of tumor growth when injected into nude mice. The results suggest that a triple-helix strategy can be used to inhibit transcription elongation of the IGF-IR gene, and emphasize the efficacy of triplex-mediated gene inhibition in an animal model.**

The type I insulin-like growth factor receptor (IGF-IR) plays an important role in the maintenance of the malignant phenotype (1). A large number of cancers and cancer-derived cell lines overexpress the IGF-IR (2). Antisense expression vectors directed against the IGF-IR have proven effective in suppressing tumor growth of C6 rat glioblastoma (3, 4), hamster mesothelioma (5), and rat prostate cancer (6). Antisense oligonucleotides (7) and the α-IR3 antibody for the IGF-IR (8–10) have also been shown to inhibit cellular proliferation in a number of cancer cell lines. Because the IGF-I receptor plays a critical role in cell proliferation and transformation, it is important to develop additional and more efficient strategies to inhibit its function.

Sequence-specific, stable triple-helical structures can be formed by hydrogen bonding of polypurine or polypyrimidine-rich oligodeoxyribonucleotides (ODNs) to polypurine tracts of double-stranded DNA *in vitro* (11–13). Thus, ODN-mediated triplex formation offers a potentially effective method for experimental or therapeutic modification of gene expression (14). Compared with antisense strategies, triplex formation targets fewer sequence copies (two versus multiple and regenerative mRNA transcripts) while maintaining high sequence specificity and stability (15, 16). ODN-mediated triplex formation can disrupt the regulation of gene expression at several points. Triplex formation was demonstrated to interfere with sequence-specific binding of transcription factors both *in vitro* and in cultured cells (see ref. 17 for review) and in addition was shown to inhibit DNA replication when directed against DNA polymerase binding sites (18–21). Reports which demonstrate that triplex formation can disrupt gene expression through inhibition of transcription elongation (22–24) suggest that it is possible to target any suitable region within the entire transcribed portion of a given gene.

The effects of exogenously supplied unmodified ODNs used to inhibit gene expression are transient since they are susceptible to extra- and intracellular degradation, thereby limiting their potential therapeutic use (25). To overcome this problem, we developed an approach in which a third strand for a potential triple helix is continuously supplied intracellularly. A recent report from our group showed that a plasmid-encoded purine oligoribonucleotide contributing a potential third strand (effector strand) for triplex formation could inhibit insulin-like growth factor type I (IGF-I) expression in stably transfected rat C6 glioblastoma cells and reduce the tumorigenicity of these cells in an animal model (26). As a target for the effector strand, we used a homopurine sequence in the promoter region of the IGF-I gene. In addition to IGF-I inhibition, a dramatic up-regulation of the serine protease inhibitor nexin-I mRNA was observed in these transfected cells.

We show here that IGF-IR transcription can be suppressed by a similar strategy. However, in this case we sought to interfere with transcription elongation by targeting potential triplex-forming oligoribonucleotides against a region of DNA encoding sequences downstream of the termination codon of the IGF-IR mRNA. To accomplish this, we designed vectors that direct transcription of the triplex-forming effector sequence of either the polypurine or polypyrimidine motif and the mRNA of a selection marker (neomycin resistance) from one promotor element as a single transcript. C6 cells transfected with the homopurine effector sequence (but not the homopyrimidine effector sequence) exhibited suppression of IGF-IR transcripts accompanied by up-regulation of nexin-I mRNA. We also observed marked down-regulation of IGF-I transcripts in these C6 cell clones. Moreover, a dramatic suppression of tumor growth in nude mice was observed, which

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Abbreviations: IGF-I, insulin-like growth factor type I; IGF-IR, IGF receptor; ODN, oligodeoxyribonucleotides.

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demonstrates that the triplex strategy can be applied as a gene therapy approach to a biological model.

MATERIALS AND METHODS

Construction of Plasmids pTH-AG-IGFIR, pTH-CT-IGFIR, and pAS-IGFIR. For assembly of plasmids appropriate for forming a triple helix with sequences in the 3′ untranslated region of the rat IGF-IR gene (accession no. L29232; from nucleotide positions 4504 to 4525), oligonucleotides were synthesized on an automated oligonucleotide synthesizer (Applied Biosystems).

The oligonucleotides IGFIR-AG (5′-GGGGTACCTCTAGAGGAAGGGAGAGAGAGGAGAGGGAATTCC-3′) and IGFIR-CT (3′-CCCCATGGAGATCTCCTTCCCTCTCTCTCCTCTCCCTTAAGG-5′) contain restriction sites for the enzymes *Eco*RI, *Xba*I, and *Kpn*I. After annealing and digestion the oligonucleotide duplexes were cloned in both orientations into a vector designated pTH-CMV (Fig. 1). This vector was derived from the eukaryotic expression vector pRc-CMV (Invitrogen), with the sequences spanning the region 995-2100 deleted by restriction digestion with the enzymes *Apa*I and *Sma*I. The resulting *Apa*I overhang was endfilled with T4 DNA Polymerase (GIBCO/BRL) according to the supplier's instructions, and the vector was religated. The digested oligonucleotides were inserted into *Eco*RI and *Kpn*I sites of vector pTH-CMV yielding the vector designated pTH-AG-IGFIR or into *Xba*I and *Eco*RI sites yielding the vector designated as pTH-CT-IGFIR. This cloning strategy positioned inserted sequences into the 5′ untranslated region of the neomycin resistance gene. Transcription of this fusion transcript is driven by the constitutive cytomegalovirus (CMV) promotor.

To prepare the antisense IGF-IR expression construct (pAS-IGFIR) a 696-bp human IGF-IR cDNA fragment (from nucleotide position 42 in exon 1 to nucleotide position 738 in exon 3) was inserted in antisense orientation into the episome-based vector pMT/EP containing the $ZnSO_4$-inducible mouse metallothionein-1 (MT-1) promotor as described previously (6).

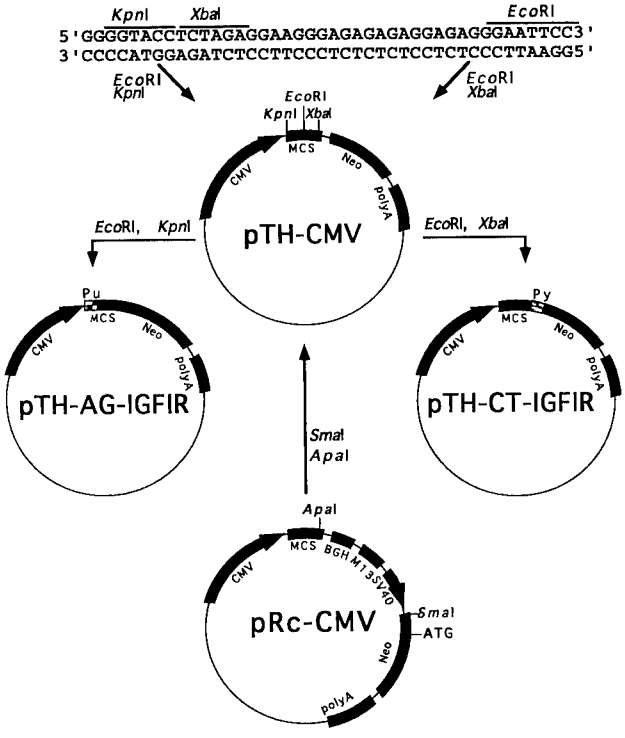

FIG. 1. Schematic representation of plasmid construction. Transcription vectors pTH-AG-IGFIR and pTH-CT-IGFIR code for the polypurine and polypyrimidine variants of triple-helix third strand, respectively. Py, polypyrimidine; Pu, polypurine; CMV, cytomegalovirus; MCS, multiple cloning site; BGH, bovine growth hormone polyadenylylation signal; M13, origin for the rescue of single strand; SV40, origin of replication; Neo, neomycin resistance gene; polyA, SV40 polyadenylylation sequence.

Cell Culture. The rat glioblastoma cell line C6 (27) was obtained from the American Type Culture Collection (Rockville, MD). To reduce intrinsic heterogeneity, we used the clone C6(t1), which was derived from parental C6 cells for experiments in this study. Cells for routine culture were maintained in Dulbecco's modified Eagle medium (BioWhittaker) supplemented with 10% fetal bovine serum (GIBCO/BRL).

Transfection. C6(t1) cells were transfected with plasmids of the vectors pTH-AG-IGFIR, pTH-CT-IGFIR, pTH-CMV (containing no insert), or pAS-IGFIR using Lipofectin (GIBCO/BRL) according to the manufacturer's instructions. Following transfection, the cells were cultured in nonselective OPTI-MEM I reduced serum medium (GIBCO/BRL) for 24 hr. Selection of pTH-AG-IGFIR, pTH-CT-IGFIR, and pTH-CMV transfectants was carried out in the presence of 0.5 mg/ml G418 (GIBCO/BRL) in the culture medium, and pAS-IGFIR transfectants were selected in medium containing 0.5 mg/ml hygromycin (Calbiochem). Clonal rings (Nalgene) were used for the isolation of single transfectants. A minimum of six single cell clones of each of the transfectants was expanded under continued selection pressure.

RNA Isolation and Hybridization. Total RNA was extracted from cells by the acid guanidine thiocyanate method (28). Poly(A)$^+$ RNA was isolated by oligothymidylated cellulose using the "messagemaker" kit (GIBCO/BRL) according to the supplier's instructions. RNA was separated on a denaturing agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham). The cDNA probes were labeled with [^{32}P]dCTP (NEN) by the random hexanucleotide primer method (29) and hybridized to Northern blots in 5× standard saline citrate (SSC), 5× Denhardt's solution, 0.1% (wt/vol) SDS and 100 μg/ml denatured salmon sperm DNA at 65°C for 18 h. The filters were washed at room temperature for 15 min in 2× SSC followed by 5–15 min in 0.5× SSC, 0.5% (wt/vol) SDS at 65°C and exposed to x-ray film for 24 h. Northern blot hybridization was carried out with a human IGF-IR cDNA insert (see above), a 160-bp portion of nexin-I cDNA (26), a 500-bp rat IGF-I cDNA fragment (30), and chicken β-actin cDNA (31) as probes.

Nude Mouse Experiments. Transfected C6(t1) cells were detached using Versene reagent (GIBCO/BRL) and washed in serum-free medium prior to injection into nude mice (HSD nu/nu, Case Western Reserve University Animal Resource Center, Cleveland). Cells (1.5×10^6 in 0.1 ml PBS) were injected subcutaneously over the right scapula of 6-week-old athymic nude mice with a 22-gauge needle. Thirteen, 12, and 10 animals were used for tumor growth assays of pTH-AG-IGFIR-, pTH-CT-IGFIR-, and pTH-CMV-transfected cells, respectively. Animals were sacrificed after 15 days and the tumors excised and weighed. Data are presented as mean ± SE.

RESULTS

A polypurine effector sequence was constructed to suppress transcription of the IGF-IR gene. This sequence was theoretically appropriate for triplex formation with a 24-base homopurine target sequence in the 3′ untranslated region of the IGF-IR gene (Fig. 2). The sequence was incorporated into the 5′ untranslated region of the neomycin resistance gene of vector pTH-CMV to maximize the probability that transfected G418-resistant cell clones would express high levels of potential triplex effector sequences (Fig. 1). C6(t1) glioblastoma cells were transfected with the resulting construct, producing several clones. Fig. 3*A* depicts a Northern blot of poly(A)$^+$ RNA from a clone (TH-AG2) transfected with vector pTH-AG-IGFIR and from untransfected cells probed with IGF-IR cDNA. A marked reduction of IGF-IR mRNA is apparent in the pTH-AG-IGFIR-transfected clone (lane 2) compared with untransfected cells

```
                          338                    360
IGF-I GENE SEQUENCE      3' TCTTCTCCCTCTCTCTCTTCC 5'
(target)                 5' AGAAGAGGGAGAGAGAGAAGG 3'
IGF-I TRIPLEX STRAND        **********************
(effector, pMT-AG-TH)    3' AGAAGAGGGAGAGAGAGAAGG 5'
                            |||x||||||||||x|||||
IGF-IR TRIPLEX STRAND    3' AAGGGAGA-GGAGAGAGAGGGAAGG 5'
(effector, pTH-AG-IGFIR)    ********-****************
IGF-IR GENE SEQUENCE     5' AAGGGAGA-GGAGAGAGAGGGAAGG 3'
(target)                 3' TTCCCTCT-CCTCTCTCTCCCTTCC 5'
                         4527                   4504
```

FIG. 2. Homology between effector strands targeted against the IGF-I gene and the IGF-IR gene. Two nucleotides (X) differ between IGF-IR and IGF-I effector strand (boldface type) sequences, which may form Hoogsteen bonds (*) with target DNA double-strand sequences. Identical nucleotide sequences are designated by |.

(lane 1). The same blot was reprobed with chicken β-actin sequences to confirm comparable RNA loading (Fig. 3*B*).

We determined levels of IGF-I transcripts (Fig. 4*A*) in pTH-AG-IGFIR-transfected cell clones as well as in two types of control cells: cells transfected with vector sequence (pTH-CMV) and cells transfected with the polypyrimidine variant of the effector sequence (pTH-CT-IGFIR; Fig. 1). As shown in Fig. 4*A*, lanes 6 and 7, IGF-I transcripts were dramatically reduced in C6(t1) cell clones TH-AG2 and TH-AG3 that were transfected with pTH-AG-IGFIR. In contrast, two C6(t1) cell clones transfected with pTH-CT-IGFIR (lanes 3 and 4) or vector (pTH-CMV; lane 2) contain IGF-I transcript levels comparable to untransfected cells (lane 1). Fig. 4*B* demonstrates integrity of loaded RNAs using chicken β-actin cDNA sequences for hybridization. These data indicate that inhibition of IGF-IR transcription by the homopurine effector sequence is accompanied by suppression of IGF-I.

To verify whether C6(t1) cells transfected with the homopurine effector sequence displayed alterations in nexin-I levels, a Northern blot of the same RNAs shown in Fig. 4*A* was carried out using nexin-I cDNA as a probe (Fig. 5*A*). RNA from two cell clones (TH-AG2 and TH-AG3) transfected with pTH-AG-IGFIR in which IGF-I transcripts were reduced showed a dramatic increase in nexin-I transcripts (lanes 9 and 10), compared with RNA from cell clones transfected with either pTH-CT-IGFIR (lanes 3–7), vector without insert pTH-CMV (lane 2), or untransfected cells (lane 1). In contrast, elevated nexin-I transcript levels were not

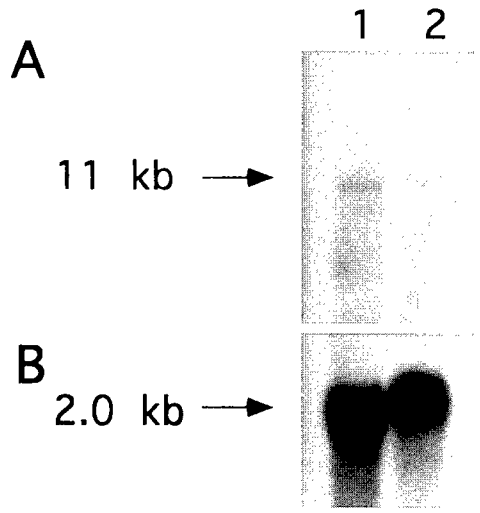

FIG. 3. Suppression of IGF-IR transcripts in C6(t1) cells transfected with pTH-AG-IGFIR construct. (*A*) Poly(A)$^+$ RNA (5 μg per lane) derived from untransfected C6(t1) cells (lane 1) and a C6(t1) cell clone (TH-AG2) transfected with the pTH-AG-IGFIR construct (lane 2) were analyzed by Northern blot. An IGF-IR cDNA was used as a probe. (*B*) Rehybridization of the same blot with chicken β-actin cDNA. Probes were labeled with ^{32}P-dCTP.

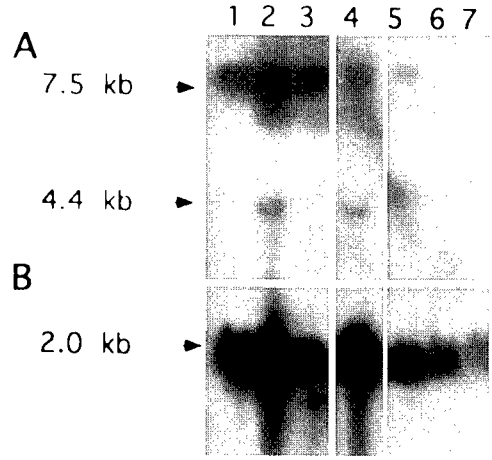

FIG. 4. Suppression of IGF-I detected by Northern blot analysis. (*A*) An IGF-I cDNA was used as a hybridization probe to analyze total RNA (20 μg per lane) from C6(t1) untransfected cells (lane 1), vector transfected cells (lane 2), cell clones TH-CT1 (lane 3), and TH-CT-2 (lane 4) transfected with pTH-CT-IGFIR, or pTH-AG-IGFIR-transfected cell clones TH-AG1 (lane 5), TH-AG2 (lane 6), and TH-AG3 (lane 7). (*B*) Rehybridization of the same filter was performed with a cDNA probe for chicken β-actin. Blots were exposed to x-ray film for 3 days.

detected in RNA from the clone TH-AG1 (Fig. 5*A*, lane 8). The IGF-I transcript level seen in clone TH-AG1 (Fig. 4*A*, lane 5) is similar to control levels and correlates with the lack of nexin-I up-regulation. These data indicate that enhanced expression of nexin-I in C6(t1) cells can act as a marker for suppression of IGF-IR as well as IGF-I. Comparable quantities of RNAs were confirmed by reprobing the same blot with chicken β-actin cDNA sequences (Fig. 5*B*).

To determine whether inhibition of IGF-IR by the polypurine triplex effector sequence affects tumor growth, nude mice were injected with transfected C6(t1) cell clones. Tumor growth was significantly decreased in all mice injected with cells transfected with pTH-AG-IGFIR (clone TH-AG2; Fig. 6*C*). Mice injected with C6(t1) cells transfected with vector pTH-CMV (Fig. 6*A*) or with C6(t1) cells transfected with

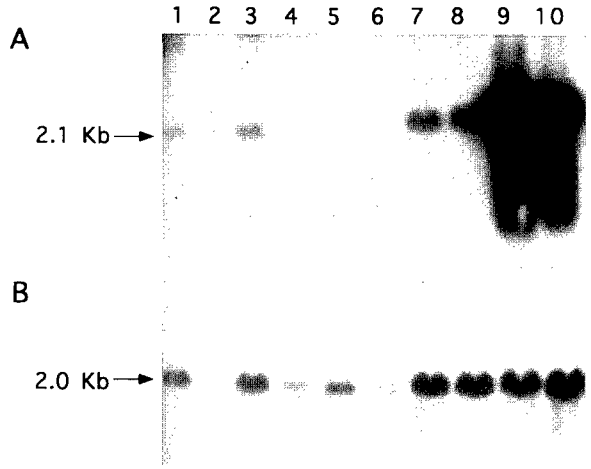

FIG. 5. Up-regulation of nexin-I transcripts detected by Northern blot analysis. (*A*) A nexin-I cDNA was used as a hybridization probe to analyze total RNA (20 μg per lane) derived from untransfected C6(t1) cells (lane 1), C6(t1) cells transfected with vector sequences only (lane 2), C6(t1) cell clones TH-CT1 through TH-CT5 (lanes 3–7) transfected with pTH-CT-IGFIR, or C6(t1) cell clones TH-AG1 (lane 8), TH-AG2 (lane 9), or TH-AG3 (lane 10) transfected with pTH-AG-IGFIR. (*B*) Rehybridization of the same filter using chicken β-actin cDNA probe.

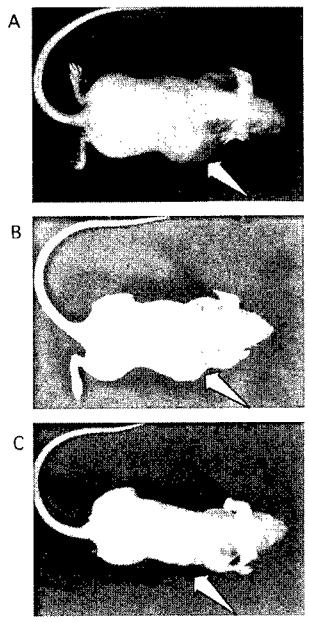

FIG. 6. Tumor growth in nude mice. Photographs of mice bearing tumors (arrows) derived from C6(t1) cells transfected with vector sequences (*A*), and from cells transfected with potential triplex-forming sequences of the pyrimidine motif (pTH-CT-IGFIR) (*B*). *C* is a photograph of a mouse injected with cells from the pTH-AG-IGFIR-transfected cell clone TH-AG2. Arrows point to regions of tumor development.

pTH-CT-IGFIR (Fig. 6*B*) bore large tumors. Fig. 7 illustrates mean weights of the tumors recovered from animals that were injected with C6(t1) cells transfected with pTH-AG-IGFIR (solid bar), pTH-CT-IGFIR (striped bar), or pTH-CMV (open bar). Tumor weights were more than 80% lower in animals injected with pTH-AG-IGFIR transfectants as compared with the other groups.

The homopurine target sequence in the IGF-IR gene contains a stretch of 19 nucleotides of which only 2 deviate from the sequence previously shown to be an effective target for inhibition of IGF-I ligand expression in C6(t1) cells (ref. 26; Fig. 2).

Therefore, the decreased levels of IGF-I transcripts observed in pTH-AG-IGFIR-transfected cells might be accounted for by triplex formation with the target sequence in the IGF-I gene. The possibility of crossreactivity of the IGF-IR effector sequence with the IGF-I gene was examined by Northern blot analysis. C6(t1) cells that had been rendered IGF-IR deficient by the independent method of antisense inhibition showed decreased levels of IGF-I

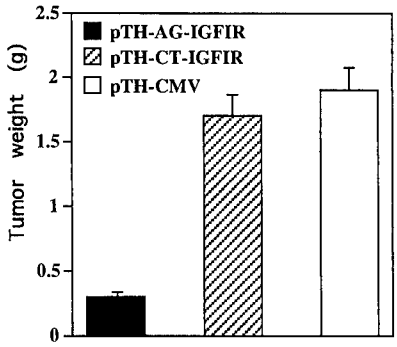

FIG. 7. Suppression of tumorigenesis by pTH-AG-IGFIR-transfected C6(t1) cells in nude mice. A total of 13, 12, and 10 nude mice were injected with 1.5×10^6 cells transfected with pTH-AG-IGFIR (solid bar), pTH-CT-IGFIR (striped bar), or vector sequences only (pTH-CMV) (open bar), respectively. Mice were sacrificed 15 days postinjection, tumors were excised, and the tumor weights were determined. Data are presented as mean ± SE.

transcripts in three different C6(t1) cell clones transfected with the antisense vector pAS-IGFIR (Fig. 8, lanes 3–5) compared with cells transfected with vector (Fig. 8, lane 2) or untransfected cells (Fig. 8, lane 1). Comparable RNA quantities were demonstrated by hybridization of the same blot with chicken β-actin sequences (Fig. 8*B*).

Crossreactivity of the IGF-I triplex effector sequence pMT-AG-TH (Fig. 2) with sequences of the IGF-IR gene was evaluated by Northern blot hybridization of RNA from C6(t1) cells rendered IGF-I deficient (Fig. 9). Inhibition of IGF-I transcript accumulation was carried out by the triple-helix strategy recently reported (26) and by antisense strategy (32). Only C6(t1) cell clones transfected with AG-triplex effector sequences against the IGF-I gene (Fig. 9*B*, lanes 3–8) or C6(t1) cells transfected with antisense to IGF-I (Fig. 9*B*, lane 2) demonstrate suppression of IGF-I. Hybridization of the blot with cDNA sequences for IGF-IR shows that IGF-IR transcript levels in IGF-I-deficient C6(t1) cells (Fig. 9*A*, lanes 2–8) are similar to those detected in IGF-I-transcribing C6(t1) cells (lanes 1 and 9). Integrity of RNA was demonstrated by hybridization of the same blot with chicken β-actin sequences (Fig. 9*C*). The data presented in Figs. 8 and 9 indicate that triplex effector sequences directed against sequences of the IGF-IR or IGF-I genes do not crossreact.

DISCUSSION

This study describes the application of a ribonucleotide sequence that can form a potential triple helix to suppress transcription of the IGF-IR gene in C6(t1) rat glioblastoma cells in culture as well as in an animal. The ability of ribonucleotide sequences to form triplexes is supported by several *in vitro* studies (33–35). Furthermore, our recent report demonstrated specific inhibition of IGF-I ligand expression by a plasmid-encoded ribonucleotide triplex effector sequence and showed a dramatic reduction in tumor growth rates in nude mice (26). In this study, it was also shown that inhibition of IGF-I expression by both plasmid-encoded antisense or potential triplex-forming ribonucleotide sequences resulted in up-regulation of nexin-I mRNA and cell surface expression of major histocompatibility complex I.

To avoid possible transcriptional repression of effector sequences due to integration site-specific inactivation (36), we employed a vector in which the triplex effector sequence and the neomycin resistance cassette were transcribed from a single promoter element. A consequence of utilizing such a

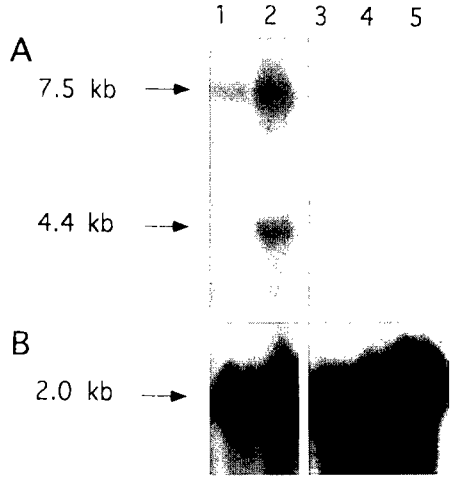

FIG. 8. Suppression of IGF-I in IGF-IR antisense-transfected C6(t1) cells. (*A*) Total RNA (20 μg per lane) derived from untransfected C6(t1) glioblastoma cells (lane 1), vector-transfected cells (lane 2), and three pAS-IGFIR-transfected cell clones (lanes 3–5) was analyzed by Northern blot hybridization using IGF-I cDNA as a probe. (*B*) Rehybridization of the same filter was performed with a cDNA probe for chicken β-actin. Blots were exposed to x-ray film for 3 days.

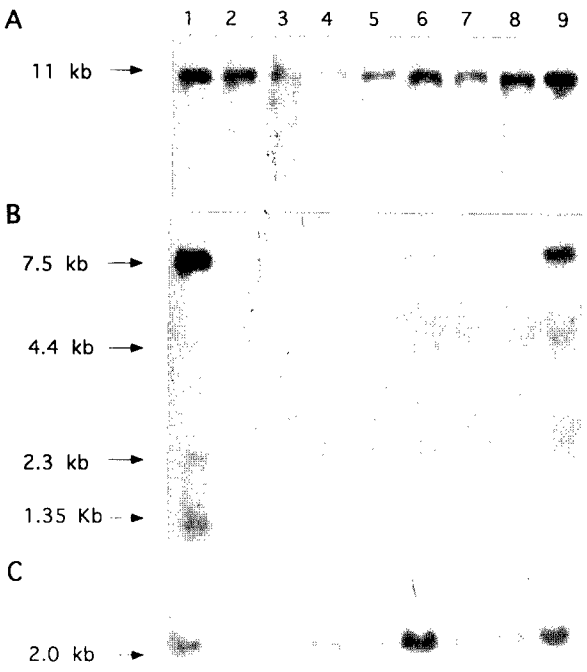

FIG. 9. Maintenance of IGF-IR transcript levels in C6(t1) cells transfected with antisense or triplex effector sequences against IGF-I. Total RNA (15 μg per lane) from vector-transfected C6(t1) rat glioblastoma cells (lane 1), antisense to IGF-I-transfected cells (lane 2), six different cell clones transfected with polypurine triplex sequences against IGF-I (lanes 3–8), or a cell clone transfected with polypyrimidine triple-helix sequences against IGF-I (lane 9) was analyzed using an IGF-IR cDNA as a hybridization probe. Rehybridizations of the same filter were performed with cDNA probes for IGF-I (*B*) or chicken β-actin (*C*).

fusion transcript is that the phenotypes of neomycin resistance and IGF-IR suppression require the transcript to be present in the nucleus as well as in the cytoplasm of a cell. Out of six clones demonstrating G418 resistance, four showed up-regulation of nexin-I mRNA, indicating that the fusion transcript was present in adequate concentrations in both nucleus and cytoplasm. When RNA from the two remaining clones was probed for neomycin resistance gene sequences, fusion transcripts were detected at reduced levels compared with RNA from the clones showing nexin-I up-regulation (unpublished observations). These results suggest that stronger selection pressure (i.e., higher G418 concentration during selection) might increase the proportion of clones with effective nuclear concentrations of triplex sequences.

C6(t1) cells transfected with pTH-AG-IGFIR exhibited dramatically suppressed tumor growth as compared with pTH-CT-IGFIR-transfected cells or cells transfected with vector sequences only, until the animals were sacrificed 2 weeks postinjection. This result strongly suggests continuous and/or stable delivery of the purine effector sequence to its nuclear target for a period of 2 weeks in the absence of selective pressure. Therefore, vector-mediated delivery of triplex-forming ribonucleotide effector sequences appears to be superior to the use of exogenously added oligonucleotides, which may lose their biological activity after several days in cultured cells (37). Furthermore, it was reported that triplexes that contain RNA rather than DNA as the Hoogsteen paired third strand are more stable, possibly through an additional hydrogen bond between the 2′ hydroxyl proton of DNA and a phosphate oxygen on the backbone of the purine RNA strand (38).

Although the structural and physicochemical properties of triplex formation have been extensively studied *in vitro*, the experimental conditions required differ considerably from those in the nucleus of an intact cell. In particular, pH constrains the formation of pyrimidine-directed triple-helical complexes, which require cytosine protonation for stabilization. Therefore, this type of triple helix is not stable at physiological conditions of pH between 7.4 and 7.8. In contrast, purine triplex structures are essentially insensitive to pH over a range of at least 5.5 to 8.3 because the proposed triplets in these structures do not involve ionized bases (39, 40). Our results are consistent with these observations since IGF-IR suppression occurred only with the polypurine triplex expression vector in C6(t1) rat glioblastoma cells. Indeed, to our knowledge, triplex formation in intact cells using unmodified ODNs has been demonstrated exclusively with guanosine-rich effector sequences (26, 37, 41–43). Pyrimidine-mediated triplex formation could be demonstrated in cells only with modified ODNs (e.g., ODNs containing a 5-methylcytosine substitution, which has been shown to reduce the stringency of pH requirements; ref. 44). Modified ODNs may possess binding affinities that are different from those of their unmodified counterparts *in vitro* (45). However, our goal was to investigate triplex formation leading to suppression of IGF-IR in a biological system.

We compared the phenotype of C6(t1) cells, in which IGF-IR was suppressed by transfection with the purine triplex expression vector pTH-AG-IGFIR, with the phenotype of cells following suppression of IGF-IR by the independent method of antisense inhibition. Both approaches resulted in down-regulation of IGF-I, the ligand of the IGF-IR. In contrast, C6(t1) cells transfected with either the pyrimidine triplex expression vector pTH-CT-IGFIR, or with control plasmids pTH-CMV (transcribing no potential triplex sequence) or pMT-EP (transcribing no antisense sequence) contained IGF-I transcript levels comparable to untransfected cells. In addition, because we observed that inhibition of IGF-I expression by either purine RNA triplex or antisense resulted in elevation of protease inhibitor nexin-I transcripts in C6(t1) cells (26), we examined pTH-AG-IGFIR-transfected and vector-transfected cells for nexin-I expression. Only C6(t1) cells transfected with the purine triplex expression vector pTH-AG-IGFIR exhibited up-regulation of nexin-I mRNA. Moreover, only C6(t1) transfectants, which showed elevated nexin-I levels, produced suppressed tumor growth in nude mice. These data, together with the fact that poly(A)$^+$ RNA from pTH-AG-IGFIR-transfected cells displayed reduced levels of IGF-IR transcripts, provide support for the specificity of triple-helix-mediated suppression of the IGF-IR gene.

The effector sequence selected to target IGF-IR gene sequences (Fig. 2) differs in 2 out of 20 nucleotides from the effector strand employed to suppress expression of the IGF-I gene, as reported recently (26). A mismatch occurs at position 6 and a deletion at position 17 relative to the IGF-IR gene sequence. Because a minimum length of 8–14 nucleotides is required for an ODN to form a triple-helical structure (17), the IGF-I effector sequence might form a partly mismatched triple-helical structure with sequences of the IGF-IR gene. However, the resulting mismatch (CG*G to CG*A, whereby "*" denotes Hoogsteen bonding) at position 6 of the IGF-I effector ribonucleotide sequence is predicted to reduce the half-dissociation temperature as indicated in the detailed study of Mergny *et al.* (46), and would be expected to disrupt stable triplex formation. Conversely, the presence of TA*G instead of TA*A at position number 6, which would occur upon binding of the IGF-IR effector strand to sequences of the IGF-I gene, would also be predicted to destabilize triplex formation. Deletion of a guanosine residue at position 17 in the IGF-IR effector sequence would be expected to further destabilize triplex formation with sequences of the IGF-I gene. The internal positions of both nucleotide deviations are predicted to be particularly disruptive to triple-helical structures, according to the study of Mergny *et al.* (46).

IGF-I effector sequence/IGF-IR target sequence crossreactivity was tested in a biological context by measuring tran-

script levels of IGF-IR in C6(t1) cells transfected with constructs encoding IGF-I triplex effector sequences. IGF-IR mRNA was not reduced in C6(t1) cells transfected with the IGF-I triplex effector sequence. These data therefore provide strong support for the specificity of the effector sequences used. We could not investigate crossreaction of the IGF-IR effector sequence with the IGF-I gene, because endogeneous IGF-I transcripts are not detectable in C6(t1) cells in which IGF-IR transcripts are repressed. As discussed above, however, the nature and position of sequence deviations between the IGF-IR effector sequence and the IGF-I target sequence suggest that such crossreaction would be unlikely to occur. In addition, the fact that suppression of IGF-IR expression in C6(t1) cells by the two independent methods of antisense and triplex both result in IGF-I deficiency argues that IGF-I suppression in IGF-IR down-regulated cells is a biological phenomenon rather than a result of crossreaction.

Because the polypurine effector strand was targeted to sequences of the IGF-IR gene that are located in the 3′ untranslated region, our results suggest that the mechanism of transcriptional inhibition is based on interruption of transcription elongation. It was shown that blocking of RNA polymerase II *in vitro* by triple-helical complexes was transient unless the triplex was stably crosslinked to the target DNA (22, 23). The authors proposed that stalling of the polymerase complex occurred in a region of a triple helix that seems to mediate triplex dissociation, allowing transcription elongation to ensue. In our experiments, we observed a low level of IGF-IR transcripts in C6(t1) cells transfected with the vector pTH-AG-IGFIR, suggesting that triple-helix-mediated suppression was not complete. An alternative explanation for the low transcript level of IGF-IR observed in pTH-AG-IGFIR-transfected cells may be that C6(t1) cells that are completely deficient in IGF-IR would fail to grow in culture since a functional IGF-IR promotes growth of C6 cells (reviewed in ref. 47).

The down-regulation of IGF-I as a consequence of IGF-IR suppression raises interesting questions. It is known that as a result of IGF-IR suppression the expression of transcription factors such as c-fos and c-jun are decreased (48–51). Possibly one of their targets may be the IGF-I gene itself. Although not the scope of this study, possible molecular mechanisms regulating the IGF-IR and its ligand IGF-I clearly await further investigation. However, our results suggest that one common mechanism could account for decreased tumorigenicity of C6(t1) glioblastoma cells (3, 32) regardless of whether IGF-I or IGF-IR expression is inhibited.

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